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The Biosynthesis of *N*-Phosphorylcreatine: an Investigation of the Postulated Alternative Pathway

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Before 1956 it was generally accepted that the sole pathway for the synthesis of *N*-phosphorylcreatine was the reaction catalysed by creatine phosphoryltransferase:

$$\text{Creatine} + \text{adenosine triphosphate} \uparrow\downarrow N\text{-phosphorylcreatine} + \text{adenosine diphosphate} \quad (1)$$

Although the results of isotopic experiments (Sacks & Altschuler, 1942; Harvey, 1955) had been interpreted as indicating the possible presence in muscle of a synthetic pathway for the formation of *N*-phosphorylcreatine not involving the adenine nucleotides, there was no direct evidence for such a reaction until the report by Cori, Abarca, Frenkel & Traverso-Cori (1956). These authors claimed that extracts of skeletal muscle catalyse the direct transfer of a phosphoryl group from 1:3-diphosphoglycerate to creatine to form *N*-phosphorylcreatine according to the equation:

$$\text{Creatine} + \text{1:3-diphosphoglycerate} \uparrow\downarrow N\text{-phosphorylcreatine} + \text{3-phosphoglycerate} \quad (2)$$

In a more detailed report (Cori, Traverso-Cori, Lagarrigue & Marcus, 1958) it was suggested that only a single enzyme was involved. Furthermore, as supporting evidence for the non-participation of adenosine triphosphate, it was stated that there was no inhibition of *N*-phosphorylcreatine synthesis in the presence of either adenosine triphosphatase or glucose plus hexokinase and that no adenine nucleotides could be detected in the reaction mixtures at the end of the incubation.

Ennor & Morrison (1958) have pointed out that because of the ubiquitous distribution of the adenine nucleotides and creatine phosphoryltransferase, any demonstration of an alternative pathway for *N*-phosphorylcreatine synthesis must unequivocally exclude creatine phosphoryltransferase. Although Cori *et al.* (1958) took rigorous precautions to exclude adenine nucleotides from the reaction mixtures, they were unable to obtain a preparation free from creatine phosphoryltransferase. However, the new reaction has received some acceptance. Thus Cohn (1959) has cited it as being an exception to the general rule that ester phosphoryl groups are transferred to form O–P bonds, and Bock (1960) has pointed out that this reaction does not fit into his classification of phosphate-transfer enzymes. Reference has been made to the reaction by Joyce & Grisolia (1959) and by Padieu & Mommaerts (1960).

In the present work the reaction has been re-examined and an attempt has been made to ascertain whether in fact creatine phosphoryltransferase is involved. No specific inhibitor of this enzyme is known and the first approach was an endeavour to separate creatine phosphoryltransferase from the enzyme responsible for catalysing reaction (2). It has been found that relatively crude preparations from rat and rabbit skeletal muscle will catalyse the overall reaction described by Cori *et al.* (1958) under conditions similar to those described by these authors. Further fractionation, however, caused a loss of activity and, since this could be recovered in greater than additive amounts by recombination of fractions, it seemed that more than one enzyme was involved. Furthermore, it has been found that diphosphopyridine nucleotide (or reduced diphosphopyridine nucleotide), which was added by the above-mentioned authors as part of the reaction system, not only functions as part of the system but also gives rise to adenosine 5′-phosphate, which is converted into adenosine triphosphate under the experimental conditions. Evidence will be presented that a direct phosphoryl-group transfer is improbable and that the reaction described probably involves the participation of adenosine di- and tri-phosphate as well as creatine phosphoryltransferase.

EXPERIMENTAL

Materials

Abbreviations. In this paper the following abbreviations will be used: ADP, adenosine diphosphate; ADP ribose, adenosine diphosphate ribose; ammediol, 2-amino-2-methylpropane-1:3-diol; AMP, adenosine 5′-phosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; $DPNH_2$, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

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Chemicals. Unless otherwise stated nucleotides and substrates were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Commercial samples of DPN (purity, 95%) and enzymically prepared $DPNH_2$ (purity, 87–90%) were tested before use, as described below (Methods), for contamination by ATP or ADP. Some samples of DPN were purified by chromatography on Dowex 1 (formate) (Kornberg, 1957) and such samples were used to prepare $DPNH_2$ by chemical reduction (Beisenherz, Bücher & Garbade, 1955). ADP ribose was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. AMP was purified by chromatography on Dowex 1 (formate). The nucleotide was eluted with 0·25N-formic acid, adsorbed on acid-washed charcoal (see below) and recovered by stirring three times with *iso*-pentyl alcohol–water (10:90, v/v). The aqueous layer from the combined extracts was evaporated to dryness under reduced pressure and the product was crystallized twice from hot water. Before use, Nuchar C and Norit A were boiled for 15 min. with N-HCl, washed free of chloride and dried at 100°.

The barium salts of fructose 1:6-diphosphate and 3-phosphoglycerate and the silver–barium salt of phosphoenolpyruvate were converted into sodium salts by adding them as slurry to the top of a column of Zeo-Karb 225 (Na^+) and washing through with water. The effluent was adjusted to pH 7·4 before making to the required volume and then treated with acid-washed charcoal. Phosphorylcreatine was prepared as the sodium salt (Ennor & Stocken, 1957). Sodium pyruvate was prepared from freshly distilled pyruvic acid, which was diluted with an equal volume of water, cooled and adjusted to pH 5 with 2N-NaOH. The sodium salt was then precipitated with acetone. Ammediol was obtained from Eastman Organic Chemicals, Rochester, N.Y., and neutralized with HCl. Hydroxylamine hydrochloride (British Drug Houses Ltd.) was neutralized before use with KOH. Calcium phosphate gel was prepared according to the method of Keilin & Hartree (1938).

Enzymes. Unless otherwise stated, frozen rabbit muscle was used as the source of the enzymes. Adenylic acid deaminase was prepared by the method of Lee (1957) to the stage of elution from calcium phosphate gel. Aldolase (Taylor, Green & Cori, 1948) and D-glyceraldehyde 3-phosphate dehydrogenase (Cori, Slein & Cori, 1948) were prepared in crystalline form. Both enzymes were twice recrystallized and stored as suspensions in 0·50 and 0·66 saturated solution of $(NH_4)_2SO_4$ respectively. Myokinase was prepared according to Colowick & Kalckar (1943), but only the 0·5–0·8 saturated $(NH_4)_2SO_4$ fraction was collected. Creatine phosphoryltransferase, prepared by method B of Kuby, Noda & Lardy (1954), was stored at 0° after freeze-drying. Before use, solutions containing 10 mg. of protein/ml. were stirred with acid-washed Norit A (1 g./10 ml.) for 15 min. at 0°, filtered and then stirred overnight with Norit A. Both treatments were then repeated. Fresh rabbit muscle was used to prepare myosin (Perry, 1955). Apyrase was prepared from potatoes by the method described by Van Thoai, Roche & An (1954) and was found not to attack AMP. Diphosphopyridine nucleotidase was prepared from mycelia of *Neurospora crassa* (Kaplan, 1955) and had an activity of 2300 units/mg. of protein. Alcohol dehydrogenase and hexokinase were commercial preparations obtained from Sigma Chemical Co. and lactic dehydrogenase was a sample from L. Light and Co. Ltd.

Rat- and rabbit-muscle preparations used in the early experiments were prepared as described by Cori *et al.* (1958) with the exception that ammediol buffer was used. Subsequently the following procedure was adopted. All operations were carried out at 2° and centrifuging was done 15 min. after the additions of $(NH_4)_2SO_4$. The muscle was treated for 2 min. in a Waring Blendor with 3 vol. of 0·01M-ammediol buffer (pH 8) and the mixture was centrifuged at 1700*g* for 20 min. The extract was filtered through cotton wool to remove fat. Ammonium sulphate (330 g./l.) was then added slowly to the filtrate with mechanical stirring. The precipitate was removed by centrifuging and $(NH_4)_2SO_4$ (155 g./l.) was added slowly with mechanical stirring to the supernatant. The precipitate was collected by centrifuging, dissolved in 0·01M-ammediol buffer, pH 8 (0·12 vol. of original extract), and dialysed against the same buffer for 16 hr. (Fraction 1). The dialysed preparation was stirred for 15 min. with Dowex 1 (Cl^-) (1 g./10 ml.) and then diluted to a protein concentration of 25 mg./ml. Ammonia solution (17N) was added to a saturated solution of $(NH_4)_2SO_4$ at 0° so that (on testing a dilution of 1 in 10) the pH was 7·4. This solution (1·5 vol.) was added dropwise with mechanical stirring to the enzyme solution obtained from the previous step and the precipitate was removed by centrifuging. The supernatant was brought then to 0·9 saturation by the slow addition of solid $(NH_4)_2SO_4$ (220 g./l.). The precipitate was collected by centrifuging, dissolved in ammediol buffer (0·4 vol. of Fraction 1). This preparation (Fraction 2) was stable for at least 2 months at 1–2°, but its stability decreased after treatment with charcoal. Fraction 1 has also been used to obtain some of the results reported in this paper and, unless otherwise stated, was stirred before use with Norit A (1 g./10 ml.) for 15 min. at 2° and filtered. Enzyme preparations have also been treated with Dowex 1 (Cl^-) (100 mg./ml.) as the loss of protein is less than that obtained as a result of treatment with charcoal.

Methods

Creatine. The release of creatine from phosphorylcreatine was estimated by the method of Rosenberg, Ennor & Morrison (1956) after stopping the enzymic reaction by the addition of an alkali–ethylenediaminetetra-acetic acid mixture (Morrison, Griffiths & Ennor, 1957).

N-Phosphorylcreatine. The formation of phosphorylcreatine was demonstrated as creatine disappearance during incubation, and verified by recovery of creatine after hydrolysis for 9 min. at 65° at pH 1–2.

Protein. This was estimated either by the biuret method of Gornall, Bardawill & David (1949) or with the Folin–Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951), crystalline bovine albumin being used as the standard for both methods.

Inorganic orthophosphate. This was determined either by the method of King (1932) or, if labile organic phosphorus compounds were present, by the method of Ennor & Stocken (1950).

3-Phosphoglycerate. This was estimated by the method of Bartlett (1959).

Ribose. This was estimated with the orcinol reaction as described by Hurlbert, Schmitz, Brumm & Potter (1954).

Diphosphopyridine nucleotide. DPN was estimated either chemically by the formation of the cyanide complex (Colowick, Kaplan & Ciotti, 1951) or enzymically with alcohol dehydrogenase by the procedure described by Bonnichsen & Brink (1955).

Reduced diphosphopyridine nucleotide. Oxidation of $DPNH_2$ was followed by studying the rate of decrease of absorption at 340 mμ in the Beckman model DK–2 ratio-recording spectrophotometer.

Nicotinamide. This was determined by measurement of the absorption at 262 mμ (ϵ $3{\cdot}1 \times 10^6$) and distinguished from nucleosides or nucleotides containing nicotinamide by the increased absorption in 0·01 N-HCl (ϵ $5{\cdot}28 \times 10^6$).

Hydroxamic acids. These were estimated by the method of Lipmann & Tuttle (1945).

Detection of low concentrations of adenine nucleotides. A linked enzyme system in which ATP and ADP act catalytically was used to detect traces of these compounds present as contaminants of other substrate nucleotide preparations. The method was also used to detect the formation of these compounds. The system involved reaction (1) and the following reaction:

$$\text{ATP} \xrightarrow{\text{myosin}} \text{ADP} + \text{inorganic phosphate}$$

The procedure was as follows: 10 μmoles of *N*-ethylmorpholine buffer, pH 7·0, 5 μmoles of $CaCl_2$, 2·5 μmoles of phosphorylcreatine and test material were incubated with 0·5 mg. of creatine phosphoryltransferase and 0·5 mg. of myosin for 20 min. at 38° in a total volume of 1·0 ml. and the release of creatine was determined. The method is capable of detecting 0·1–0·2 μm-mole of ATP, and is ten times as sensitive as the firefly-luminescence-assay method (Strehler & Totter, 1954) or the catalytic linked system, involving hexokinase and glucose 6-phosphate dehydrogenase, which was used by Cori *et al.* (1958). There was some inhibition of the release of creatine with added ATP at concentrations of AMP or DPN above 2·5 mM and this limited the assay to the detection of 1 part of ATP or ADP in 2500 parts of those compounds. No ATP or ADP could be detected in the purified samples of DPN and AMP, or in the commercial DPN used in this work. However, the commercial $DPNH_2$ and ADP ribose were contaminated by ATP or ADP to the extent of 0·1–0·2% and 0·05–0·1% respectively.

Assay of adenine nucleotide. The determination of AMP and ATP plus ADP was carried out with adenylic acid deaminase and potato apyrase (Kalckar, 1947) and the changes in extinction were followed in a Beckman DK–2 ratio-recording spectrophotometer in the presence of 0·1 M-sodium succinate buffer (pH 6·5).

Chromatography and electrophoresis. Nucleotides and nicotinamide were chromatographed on Whatman 3 MM paper by ascending chromatography in either (*a*) *iso*butyric acid–aq. NH_3 soln. (sp.gr. 0·88)–water (66:1:33, by vol.) or (*b*) ethanol–1 M-ammonium acetate, pH 7·5 (7:3, v/v) and were detected by visual inspection in ultraviolet light. Nicotinamide and its derivatives were also detected by the methods described by Kodicek & Reddi (1951). Nicotinamide and DPN could be rapidly separated (2–3 hr.) by electrophoresis either in 0·033 M-borate buffer, pH 8·9, or 0·033 M-phosphate buffer, pH 7·0 (Sundaram, Rajagopalan & Sarma, 1959). Dinitrophenylhydrazones were chromatographed by ascending chromatography in (*c*) 2-methylbutan-2-ol–ethanol–water (50:10:40, by vol.) (Ichihara & Greenberg, 1957) or by descending chromatography in (*d*) butan-1-ol–N-$NaHCO_3$ (1:2, v/v) with bicarbonate-washed papers (Seligson & Shapiro, 1952). Free acids were separated by descending chromatography in (*e*) pentan-1-ol–5 M-formic acid (1:1, v/v) (Buch, Montgomery & Porter, 1952) and detected by spraying with bromophenol blue. Hydroxamic acids were chromatographed by descending chromatography in (*f*) butan-2-ol–formic acid–water (75:15:10, by vol.) (Hoagland, Keller & Zamecnik, 1956), (*g*) ethyl acetate–acetone–water (1:2:2·75, by vol.) (Micheel & Albers, 1956) and (*h*) butan-1-ol–acetic acid–water (4:1:5, by vol.) (Thompson, 1951) and were detected by the method of Lipmann & Tuttle (1945).

RESULTS

Because the release of creatine from phosphorylcreatine can be determined more readily than can the formation of phosphorylcreatine from creatine, the initial studies were concerned with the reaction between phosphorylcreatine and 3-phosphoglycerate to form creatine and 1:3-diphosphoglycerate. It was pointed out by Cori *et al.* (1958) that this approach suffers from the disadvantage of the unfavourable equilibrium but that this can be overcome by the addition of triose phosphate dehydrogenase, $DPNH_2$ and arsenate. This results in arsenolysis of the 1:3-diphosphoglycerate formed and thus increases the release of creatine according to the following reactions:

$$\text{phosphorylcreatine} + \text{3-phosphoglycerate} \rightleftarrows \text{1:3-diphosphoglycerate} + \text{creatine}$$

$$\text{1:3-diphosphoglycerate} + \text{water} \xrightarrow[\text{arsenate}]{DPNH_2,\ \text{triose phosphate dehydrogenase}} \text{3-phosphoglycerate} + \text{inorganic phosphate}$$

Table 1. *Requirements for the release of creatine from phosphorylcreatine in the presence of 3-phosphoglycerate*

Complete reaction mixture contained: ammediol buffer (pH 7·4), 100 μmoles; 3-phosphoglycerate, 4 μmoles; phosphorylcreatine, 2·5 μmoles; Mg^{2+} ion, 5 μmoles; $DPNH_2$, 0·25 μmole; arsenate, 1 μmole; enzyme, 2 mg. of a 0·64–0·75 sat. ammonium sulphate fraction of rabbit-muscle extract prepared as described by Cori, Traverso-Cori, Lagarrigue & Marcus, (1958). Volume, 1·0 ml.; temp., 38°; reaction time, 15 min. The enzyme preparation was stirred at 2° for 15 min. with Norit A (1 g./10 ml.) and filtered. The filtrate was stirred overnight with a similar amount of charcoal and again filtered.

Components	Creatine released (μmole/ml.)
Complete	0·315
Without arsenate	0·125
Without $DPNH_2$	0
Without 3-phosphoglycerate	0
Without Mg^{2+} ion	0·045

With the additions referred to above, our results were similar to those reported by Cori *et al.* (1958). Thus charcoal-treated enzyme preparations from

either rat or rabbit skeletal muscle catalysed the release of creatine from phosphorylcreatine. The release of creatine required the presence of 3-phosphoglycerate and $DPNH_2$ and was increased by the addition of arsenate and Mg^{2+} ions (Table 1). In the present experiments, the rate of reaction was linear over the first 30 min. and was not affected by the addition of triose phosphate dehydrogenase, which, as was subsequently shown, was present in all muscle preparations. The rate of release of creatine from phosphorylcreatine was dependent upon the concentration of $DPNH_2$, and purified DPN was far less effective than $DPNH_2$ (Table 2). Since triose phosphate dehydrogenase catalyses equally well the arsenolysis of 1:3-diphosphoglycerate in the presence of either DPN or $DPNH_2$ (Racker & Krimsky, 1952), this result suggests that if 1:3-diphosphoglycerate is formed, then its formation is dependent on the presence of $DPNH_2$ and therefore probably does not occur by direct phosphoryl-group transfer.

In the absence of arsenate, $DPNH_2$ was oxidized when incubated with the enzyme preparation, 3-phosphoglycerate and phosphorylcreatine. The rate of this reaction was linear, as also was the release of creatine, but the amount of $DPNH_2$ oxidized at any time was greater than the amount of creatine released (Table 3). According to the reaction postulated by Cori *et al.* (1958) the amount of creatine released should be equivalent to the 1:3-diphosphoglycerate formed and, if the oxidation of $DPNH_2$ were due to the reaction 1:3-diphosphoglycerate + $DPNH_2$ → 3-phosphoglyceraldehyde + inorganic phosphate + DPN, the amount of $DPNH_2$ oxidized could not exceed the amount of creatine released.

Table 2. *Effect of oxidized and reduced diphosphopyridine nucleotide on the release of creatine from phosphorylcreatine in the presence of 3-phosphoglycerate*

Reaction mixtures contained: ammediol buffer (pH 7·4), 100 μmoles; 3-phosphoglycerate, 4 μmoles; phosphorylcreatine, 2 μmoles; Mg^{2+} ion, 5 μmoles; arsenate, 1 μmole; enzyme, 4·2 mg. of (A) and 5·6 mg. of (B), where A and B represent different preparations of Fraction 1 treated with charcoal as described in Table 1. Total volume, 1·0 ml. Tubes were incubated for 15 min. at 38°.

Nucleotide	Concn. (mM)	Creatine released (μmole/ml.) A	B
DPN (purified)	0·25	—	0·027
	0·1	—	0·010
$DPNH_2$	0·25	0·323	0·380
	0·1	0·144	0·160
	0·05	0·082	—
	0·025	0·043	—
	0·01	0·018	—
$DPNH_2$ (prepared chemically from purified DPN)	0·25	—	0·35
	0·10	—	0·19

Table 3. *Relationship between the oxidation of reduced diphosphopyridine nucleotide and the release of creatine from phosphorylcreatine in the presence of 3-phosphoglycerate*

Reaction mixture contained: ammediol buffer (pH 7·4), 0·75 m-mole; phosphorylcreatine, 7·5 μmoles; Mg^{2+} ion, 15 μmoles; cysteine, 60 μmoles; 3-phosphoglycerate, 12 μmoles; $DPNH_2$, 0·3 μmole; enzyme, 1·2 mg. of a 0·6–0·9 sat. ammonium sulphate fraction of muscle extract treated with charcoal as described in Table 1. The total volume was 3 ml. and the reaction mixture was incubated in spectrophotometer cells at 18°.

Incubation time (min.)	Creatine released (μmole/ml.)	DPN formed (μmole/ml.)	$DPNH_2$ oxidized (%)
10	0·029	0·05	52
20	0·058	0·096	100

Evidence for the requirement of more than one enzyme for reaction (2)

When the enzyme preparation (Fraction 1) was subjected to further fractionation with calcium phosphate gel, acetone or ethanol, the specific activity of each fraction obtained was less than that of the original solution. (The enzymic activity was determined by measuring the rate of creatine release from phosphorylcreatine in the presence of 3-phosphoglycerate, DPN, triose phosphate dehydrogenase and arsenate.) As activity could be recovered in more than additive amounts by recombination of two or more fractions, it seemed that more than one enzyme was involved and that the mechanism proposed by Cori *et al.* (1958) was probably incorrect.

If a direct phosphoryl-group transfer were operative, it would be expected that when phosphorylcreatine, 3-phosphoglycerate and hydroxylamine were incubated with the enzyme preparation (Fraction 1) a hydroxamic acid derivative of 1:3-diphosphoglycerate would be formed. However, under these conditions, no such derivative could be detected. The failure to obtain a hydroxamate was not due to the high KCl concentration associated with the hydroxylamine, for an equivalent amount of KCl did not inhibit the release of creatine with added $DPNH_2$. It is not known whether or not creatine was released in the presence of hydroxylamine because hydroxylamine interfered with the colorimetric determination of creatine.

The addition of $DPNH_2$ (0·25 mM) to the reaction mixture caused the formation of a hydroxamate, which was also obtained in the absence of phosphorylcreatine. The reaction could not be attributed to the ADP or ATP content (0·1–0·2 %) of the $DPNH_2$ used, for these adenine nucleotides were inactive at 1 μM, although activity was obtained at

higher concentrations. When an enzyme preparation twice treated with charcoal was used, $DPNH_2$ could not be replaced by DPN, although the latter was active after chemical reduction (cf. Table 2).

Addition of DPN did bring about hydroxamate formation with a preparation treated once with charcoal. The reason for this difference is not known, but it may be related to the further removal of protein by a second charcoal treatment.

Reactions of 3-phosphoglycerate catalysed by the muscle preparation in the absence of phosphoryl-creatine

The hydroxamic acid derivative formed in the presence of 3-phosphoglycerate and either DPN or $DPNH_2$, in the absence of phosphoryl-group donors, was obtained under the conditions described in Table 4. As the enzyme preparation also contained 3-phosphoglycerate kinase, the hydroxamic acid derivative of 1:3-diphosphoglycerate was prepared under the same conditions with ATP (20 mM) replacing the $DPNH_2$ or DPN. At the end of the incubation period (2 hr.) an equal volume of ethanol was added and the mixtures were boiled. Cations were removed from the supernatant solution by addition of Zeo-Karb 225 (H^+ form). The hydroxamates formed from 3-phosphoglycerate in the presence of DPN, $DPNH_2$ or ATP showed identical behaviour when chromatographed in solvents (*f*), (*g*) and (*h*), and the presence of phosphate in each was demonstrated after chromatography in solvent (*f*). In this solvent there was a clear separation of the hydroxamate (R_F 0·1) from inorganic phosphate (R_F 0·5). It was thus concluded that the muscle preparations formed 1:3-diphosphoglycerate in the presence of 3-phosphoglycerate and either $DPNH_2$ or DPN. This could be explained by the conversion of some 3-phosphoglycerate into phosphoenolpyruvate, which would then act as a phosphoryl-group donor. Such an explanation is supported by the finding that hydroxamate formation with either DPN or $DPNH_2$ was inhibited by fluoride in the presence, but not in the absence, of phosphate (Table 4). The probable involvement of phosphoenolpyruvate was also shown by the disappearance of 3-phosphoglycerate in the absence of pyridine nucleotides (cf. Table 6) and the formation of an iodine-labile phosphorus compound (Schmidt, 1957).

The transfer of a phosphoryl group from phosphoenolpyruvate to 3-phosphoglycerate would result in the formation of pyruvate in addition to 1:3-diphosphoglycerate. That such a reaction did occur was confirmed by the isolation of pyruvate from those reaction mixtures which contained DPN or $DPNH_2$. The identity of the isolated material as pyruvate was confirmed by the similarity in chromatographic behaviour of the free acid and its 2:4-dinitrophenylhydrazone with that of an authentic sample of pyruvate and its hydrazone (see the Methods section). In addition, the melting point (214–215°) of the 2:4-dinitrophenylhydrazone derivative was identical with that of the authentic compound and was unchanged by admixture with the latter.

In the presence of pyridine nucleotides 3-phosphoglycerate gave rise to inorganic phosphate (Fig. 1). Although some phosphatase activity is apparent, the addition of DPN or $DPNH_2$ has a marked effect on the release of inorganic phosphate. The kinetics of the release of inorganic phosphate show that the initial rate is higher with $DPNH_2$ than with DPN and are consistent with inorganic phosphate being derived from 1:3-diphosphoglycerate.

Table 4. *Effect of fluoride on the formation of hydroxamate from 3-phosphoglycerate in the presence of oxidized and reduced diphosphopyridine nucleotide*

Reaction mixtures contained: nucleotide, 1 μmole; 3-phosphoglycerate, 8 μmoles; Mg^{2+} ion, 5 μmoles; hydroxylamine, 0·2 m-mole; enzyme, 3·5 mg. of Fraction 1; either phosphate buffer (pH 7·4), 50 μmoles or ammediol buffer (pH 7·4), 100 μmoles; total volume, 1 ml. After 1 hr. at 38°, the apparent hydroxamate formation was determined by the method of Lipmann & Tuttle (1945). Results were calculated from the molecular-extinction value for the hydroxamate of 1:3-diphosphoglycerate as quoted by Krimsky (1959). Inhibition of the colour formation by components of the reaction mixtures and added fluoride ions was studied with succinic anhydride as a standard.

Buffer	Nucleotide	Fluoride added (μmoles)	Apparent hydroxamate formation (μmoles/ml.)	Inhibition of the colour formation (%)	Corrected hydroxamate formation (μmoles/ml.)
Phosphate	DPN	—	1·62	31·5	2·37
	$DPNH_2$	—	1·71	—	2·51
	DPN	5	0·303	33·0	0·452
	$DPNH_2$	5	0·176	—	0·262
	DPN	10	0·016	34·5	0·024
	$DPNH_2$	10	0	—	0
Ammediol	$DPNH_2$	—	2·08	17·0	2·51
	$DPNH_2$	5	2·25	18·3	2·79

This compound decomposes spontaneously at pH 7·4 and 38°, but in the presence of triose phosphate dehydrogenase and arsenate a much more rapid breakdown occurs.

The conversion of 3-phosphoglycerate into 1:3-diphosphoglycerate, involving phosphoenolpyruvate, which occurs in the presence of DPN (or $DPNH_2$) and the muscle preparation, would also be catalysed by the enzymes of the glycolytic cycle in the presence of ADP or ATP. That the appropriate glycolytic enzymes were present in the muscle preparation was demonstrated by the fact that there was a release of inorganic phosphate from 3-phosphoglycerate when $DPNH_2$ was replaced by either ADP or ATP (Table 5). The greater effectiveness of $DPNH_2$ can be explained by the requirement of triose phosphate dehydrogenase for either DPN or $DPNH_2$ in the arsenolytic reaction.

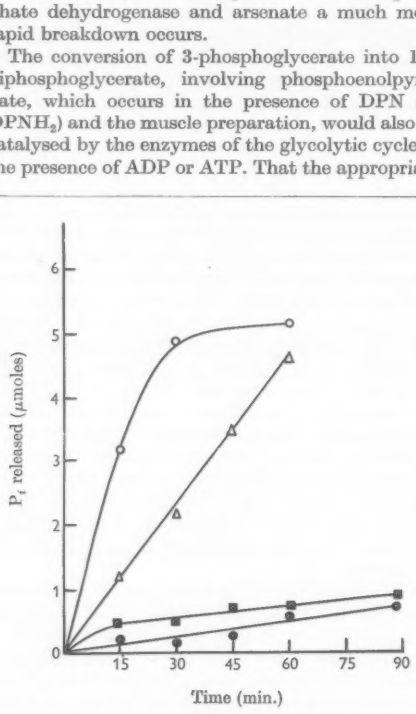

Fig. 1. Rate of release of inorganic phosphate (P_i) from 3-phosphoglycerate on the addition of oxidized and reduced diphosphopyridine nucleotide in the presence and absence of arsenate. Reaction mixtures contained: 3-phosphoglycerate, 5·5 μmoles; Mg^{2+} ion, 5 μmoles; ammediol buffer (pH 7·4), 200 μmoles; enzyme, 4 mg. of Fraction 1 in a total volume of 0·66 ml. Temp., 38°. Additions were: ○, $DPNH_2$ (1 μmole) and arsenate (1 μmole); △, DPN (1 μmole) and arsenate (1 μmole); ■, $DPNH_2$ (1 μmole); ●, DPN (1 μmole).

Reactions between phosphorylcreatine and 3-phosphoglycerate in the presence of oxidized and reduced diphosphopyridine nucleotide

Hydrolysis of phosphorylcreatine. A comparison of the amounts of inorganic phosphate formed from 3-phosphoglycerate and from 3-phosphoglycerate plus phosphorylcreatine (Table 6) shows that the increased amount of inorganic phosphate formed in the presence of phosphorylcreatine is equivalent to the amount of creatine released, indicating overall hydrolysis of phosphorylcreatine. In both cases the release of inorganic phosphate is markedly increased upon the addition of pyridine nucleotides and all the 3-phosphoglycerate disappears. In the absence of pyridine nucleotides there is also a loss of 3-phosphoglycerate, which is due to the formation of phosphoenolpyruvate and presumably 2-phosphoglycerate.

Table 5. *Effect of reduced diphosphopyridine nucleotide and adenine nucleotides on the release of inorganic phosphate from 3-phosphoglycerate*

Reaction mixtures contained: 3-phosphoglycerate, 8 μmoles; Mg^{2+} ion, 5 μmoles; ammediol buffer (pH 7·4), 100 μmoles; arsenate (pH 7·4), 1 μmole; nucleotide, 1 μmole; enzyme, 4 mg. of Fraction 1; volume, 0·56 ml. Tubes were incubated at 38°.

Nucleotide added	Inorganic phosphate released (μmole/tube)	
	15 min.	35 min.
None	0·05	0·067
$DPNH_2$	0·272	0·381
ADP	0·128	0·239
ATP	0·127	0·159

Table 6. *Release of inorganic phosphate from 3-phosphoglycerate in the presence and absence of phosphorylcreatine*

Reaction mixtures contained: 3-phosphoglycerate, 4 μmoles; Mg^{2+} ion, 2·5 μmoles; ammediol buffer (pH 7·4), 100 μmoles; arsenate (pH 7·4), 0·5 μmole; enzyme, 2 mg. of Fraction 1. Additions were: DPN, 0·5 μmole; $DPNH_2$, 0·5 μmole; phosphorylcreatine, 2·5 μmoles. Volume, 0·4 ml.; temp., 38°; incubation time, 3 hr.

	Phosphorylcreatine absent			Phosphorylcreatine present			
		Δ			Δ		
Additions	Inorganic phosphate (μmoles)	Inorganic phosphate (μmoles)	3-Phosphoglycerate (μmoles)	Inorganic phosphate (μmoles)	Inorganic phosphate (μmoles)	Creatine (μmoles)	3-Phosphoglycerate (μmoles)
None	0·33	—	1·28	0·76	—	0	1·28
$DPNH_2$	3·63	3·3	0	5·28	4·5	1·17	0
DPN	3·75	3·4	0	5·68	4·9	1·47	0

When 3-phosphoglycerate was replaced by pyruvate there was a release of creatine from phosphorylcreatine, but only in the presence of DPN or $DPNH_2$ (Table 7). The amount of creatine released in 20 min. was greater in the presence of $DPNH_2$ than in the presence of DPN. The addition of arsenate increased the rate of both reactions and this finding suggests that 1:3-diphosphoglycerate is formed.

The results indicate that either $DPNH_2$ and DPN may in some way be functioning as a source of ADP or ATP, or the pyridine nucleotides may be acting in a novel way as phosphoryl-group carriers. It is unlikely, on energy grounds, that the pyridine nucleotide phosphoryl carrier would be TPN. Moreover, although the addition of TPN resulted in a release of creatine in the presence of 3-phosphoglycerate, the reaction rate was slower than with an equivalent amount of DPN.

Tests were carried out to determine if DPN and $DPNH_2$ underwent enzymic hydrolysis at pH 7·4. The results were negative, as judged by assay with both the cyanide and alcohol-dehydrogenase procedures, although the methods could not be relied upon to detect less than 3% of hydrolysis.

Table 7. *Effect of arsenate on the release of creatine from phosphorylcreatine in the presence of pyruvate*

Reaction mixtures contained: phosphorylcreatine, 2·5 μmoles; Mg^{2+} ion, 5 μmoles; ammediol buffer (pH 7·4), 100 μmoles; sodium pyruvate, 10 μmoles; enzyme, 4·5 mg. of Fraction 1 treated with Dowex 1 (Cl^-) (1 g./10 ml.). Additions were: arsenate (pH 7·4), 1 μmole; DPN, 0·5 μmole; $DPNH_2$, 0·5 μmole. Total volume, 1 ml. Creatine was determined after 20 min. at 38°.

Additions	Creatine released (μmole/ml.)
None	0
DPN	0·055
DPN + arsenate	0·171
$DPNH_2$	0·176
$DPNH_2$ + arsenate	0·600

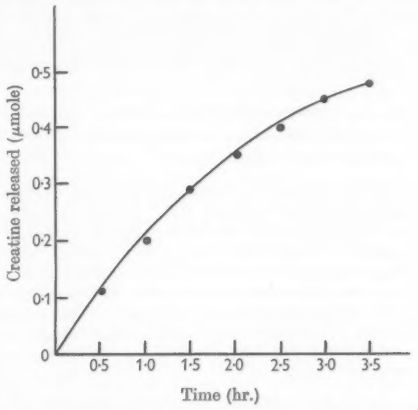

Fig. 2. Rate of release of creatine from phosphorylcreatine in the presence of diphosphopyridine nucleotide. Reaction mixture contained: ammediol buffer (pH 8·5), 100 μmoles; Mg^{2+} ion, 5 μmoles; phosphorylcreatine, 5 μmoles; DPN (Sigma; purity, 95%), 2·5 μmoles; enzyme, 8·3 mg. of Fraction 2. Volume, 1 ml., temp., 38°.

Table 8. *Stability of diphosphopyridine nucleotide under conditions leading to the diphosphopyridine nucleotide-dependent release of creatine from phosphorylcreatine*

Reaction mixtures contained: ammediol buffer (pH 8·5), 100 μmoles; Mg^{2+} ion, 5 μmoles; DPN, 2·5 μmoles. Additions were: phosphorylcreatine, 5 μmoles; apyrase, 33 μg.; enzyme, 7 mg. of Fraction 2 (Expt. 1) or 8 mg. of Fraction 1 (Expt. 2). Volume, 1 ml.; temp., 38°; incubation time, 4 hr. (Expt. 1) or 2 hr. (Expt. 2). For the DPN assays 0·15 ml. samples were taken and the final volume in each case was 3·6 ml.

		DPN assay			
		Cyanide method ($E_{325\,m\mu}$)		Enzymic method ($E_{340\,m\mu}$)	
Additions	Creatine released (μmoles/ml.)	With enzyme	Without enzyme (or boiled enzyme)	With enzyme	Without enzyme (or boiled enzyme)
Expt. 1					
None	—	0·578	0·583	0·592	0·602
Phosphorylcreatine	0·60	0·583	0·583	0·598	0·605
Expt. 2					
None	—	0·560	—	0·572	—
Phosphorylcreatine	0·48	0·554	—	0·570	—
Apyrase	—	0·554	—	0·564	
Apyrase + phosphorylcreatine	5·0	0·560	—	0·565	—

Release of creatine from phosphorylcreatine in the presence of diphosphopyridine nucleotide

If the pyridine nucleotides were acting as phosphoryl-group carriers, creatine (or pyruvate) should be released when phosphorylcreatine (or phosphoenolpyruvate) is incubated in the presence of the muscle preparation with substrate amounts of DPN. Creatine was released under these conditions (Fig. 2), with no detectable release of inorganic phosphate or enzymic destruction of DPN. (DPN, rather than $DPNH_2$, was used for these experiments as it was known to be free of contaminating adenine di- and tri-phosphate.) The addition of apyrase, either in the presence or absence of phosphorylcreatine, did not bring about any detectable destruction of DPN (Table 8) but there was a quantitative release of creatine and inorganic phosphate from phosphorylcreatine that was dependent upon DPN. The amount of creatine released was affected by pH and DPN concentration (Table 9) but not by the Mg^{2+}-ion concentration.

These results are consistent with the formation of a phosphorylated derivative of DPN, which could be hydrolysed by apyrase, and efforts were directed towards the isolation of the compound.

The fractionation on Dowex 1 (formate) of the reaction mixture, after DPN and phosphorylcreatine were incubated with the muscle preparation at pH 8·5, was carried out as described in Table 10. The effluent from the Dowex 1 column was free of ribose and was identified spectrophotometrically and chromatographically as containing nicotinamide. Fraction 1 was identified chromatographically as containing DPN and treatment of this fraction with apyrase did not give rise to inorganic phosphate. Fraction 2 was free of acid-labile phosphate and was shown to contain adenine : ribose : phosphate in the ratio 1:2·02:1·8, indicating that the compound was ADP ribose. A comparison of the paper-chromatographic behaviour of this compound in solvent systems (*a*) and (*b*) with that of an authentic sample of ADP ribose showed that the two compounds were identical. The presence of ATP in Fraction 3 was shown by paper chromatography and enzymic analysis. Thus the amount of inorganic phosphate released by myosin was half that released by apyrase and by myosin plus myokinase. The product formed by the action of apyrase was AMP as judged by analysis with adenylic acid

Table 9. *Factors affecting the release of creatine from phosphorylcreatine in the presence of diphosphopyridine nucleotide*

Reaction mixtures (A) contained: Mg^{2+} ion, 5 μmoles; phosphorylcreatine, 5 μmoles; DPN, 2·5 μmoles; enzyme, 14 mg. of Fraction 2; ammediol buffer (required pH), 100 μmoles. Volume, 1 ml.; temp., 38°. Creatine was determined at the end of a 3 hr. incubation period and the pH of duplicate tubes was checked after the same period. Results reported under B were obtained under the same conditions as described for A with the exception that ammediol buffer (pH 8·5) was used and the DPN concentration was varied.

A		B	
pH	Creatine released (μmole/ml.)	DPN (μmoles/ml.)	Creatine released (μmole/ml.)
7·4	0·45	3·5	0·74
7·8	0·49	2·5	0·60
8·4	0·60	2·0	0·49
8·7	0·68	1·5	0·38
		1·0	0·29
		0·5	0·13
		0·25	0·10

Table 10. *Products formed as a result of the incubation of diphosphopyridine nucleotide with phosphorylcreatine*

Incubation mixture contained: DPN, 136 μmoles; phosphorylcreatine, 250 μmoles; ammediol buffer (pH 8·5), 4 m-moles; Mg^{2+} ion, 200 μmoles; enzyme, 166 mg. of Fraction 2 in a volume of 40 ml. The mixture was incubated for 2 hr. at 38° and the release of creatine was 0·31 μmole/ml. The reaction mixture was passed through a column of Nuchar C (3 g.), which was washed consecutively with 100 ml. of water, 60 ml. of 0·01 N-ethylenediaminetetra-acetic acid (pH 7·0) and 100 ml. of water. Nucleotides were eluted with 250 ml. of *iso*pentyl alcohol–water–ethanol (10:40:50). The organic solvent was removed under reduced pressure and the solution freeze-dried. The solids were redissolved in 50 ml. of water, adjusted to pH 8·5 by addition of N-NaOH and fractionated on Dowex 1 (formate) at 2°. The fractions were recovered from the eluting solution by adsorption on and elution from Nuchar C as described above.

Fraction	Eluting solution	Product identified	Recovery (% of total $E_{260\,m\mu}$ material)
Effluent	Water	Nicotinamide	0·6
1	Formic acid (0·1 N)	DPN	81·0
2	Formic acid (4 N)	ADP ribose	10·5
3	Formic acid (4 N)–ammonium formate (0·4 N)	ATP	3·5

deaminase. Fraction 3 also contained, apart from ATP, some material which showed absorption at 260 mμ, but no attempt was made to isolate or identify this material.

The ATP could have arisen in three ways: by hydrolysis of a phosphorylated derivative of DPN during the fractionation procedure, or from contamination of the commercial DPN by compounds which can give rise to ATP under the experimental conditions or by non-enzymic hydrolysis of DPN to AMP with subsequent phosphorylation of the latter by phosphorylcreatine. To determine if ATP arose as a result of the breakdown of a phosphorylated derivative of DPN, incubation mixtures were analysed for ATP after incubation of DPN and phosphorylcreatine with the enzyme preparation for 2 hr. under the conditions described in Table 10. The high concentration of DPN precluded accurate analysis with the myosin–creatine phosphoryltransferase system, but accurate analysis could be obtained with adenylic acid deaminase and apyrase, as these enzymes are unaffected by the presence of DPN. The results showed that ATP or ADP was formed in amounts equivalent to about 10 % of the added DPN. This assay also showed that AMP was formed in the absence of phosphorylcreatine and that the amount was equivalent to the ADP or ATP formed when phosphorylcreatine was present. If the muscle-enzyme preparation were omitted, there was no AMP formation, so the latter must have arisen enzymically during the incubation. The formation of AMP in the absence of phosphorylcreatine and of ATP in the presence of phosphorylcreatine was confirmed by chromatographic and enzymic analysis of the products isolated under the following conditions: at the end of the incubation period, the reaction mixture was heated for 1·5 min. in a boiling-water bath, cooled and the protein removed by centrifuging. To the supernatant was added 0·3 ml. of barium acetate (2M) and 20 ml. of absolute ethanol. After standing for 30 min., the precipitate of barium salts was collected by centrifuging and dried in a vacuum desiccator. The barium salts were converted into sodium salts by passage through a column of Zeo-Karb 225 (Na^+) as described above. (Assay of the supernatant solution with adenylic acid deaminase and apyrase showed that there was no increase in the total adenine nucleotides as a result of boiling the reaction mixture for 1·5 min.)

Since no enzymic hydrolysis of DPN could be detected in the reaction mixture it thus seemed that the AMP must have arisen from a contaminant of the DPN sample. Such a contaminant could be ADP ribose. The DPN used in these experiments had not been purified, for it had been shown not to be contaminated with ATP, ADP or AMP as judged by analysis with creatine phosphoryltransferase plus myosin and with adenylic acid deaminase. However, such tests would not give any indication of whether or not ADP ribose was present. When this compound was added the results were similar to those obtained in the presence of DPN (Table 11). The addition of $DPNH_2$ and TPN also gave essentially the same results. The yield of AMP in the absence of phosphorylcreatine and the yield of ATP and creatine in the presence of phosphorylcreatine were approximately the same on the addition of DPN, $DPNH_2$ and TPN, but were very much greater with an equivalent amount of ADP ribose. Under the same conditions, but with the amount of ADP ribose reduced to 0·3 μmole, it was shown by chromatography that this compound completely disappeared. At the same time, 0·6 μmole of creatine was released from phosphorylcreatine. (Because of the specificity of adenylic acid deaminase, the formation of AMP from TPN suggests that the phosphoryl group on the 3′ position of the ribose moiety has been removed by hydrolysis.)

The above-mentioned results with ADP ribose could be accounted for by the reactions:

ADP ribose → ribose 5′-phosphate + AMP

AMP + 2 phosphorylcreatine → ATP + 2 creatine.

Table 11. *Formation of adenine nucleotides from pyridine nucleotides and adenosine diphosphate ribose*

Reaction mixtures contained: ammediol buffer (pH 8·5), 100 μmoles; Mg^{2+} ion, 5 μmoles; nucleotide, 2·5 μmoles; enzyme, 7·2 mg. of Fraction 2; where indicated, phosphorylcreatine, 5 μmoles. Volume, 1 ml.; temp., 38°; incubation time, 4 hr.

Nucleotide	Additions	AMP (μmole/ml.)	ADP or ATP (μmole/ml.)	Creatine (μmole/ml.)
DPN (non-purified)	None	0·30	—	—
	Phosphorylcreatine	—	0·30	0·65
$DPNH_2$	None	0·31	—	—
	Phosphorylcreatine	(Trace)	0·28	0·68
TPN	None	0·348	—	—
	Phosphorylcreatine	—	0·33	0·68
ADP ribose	None	0·89	—	—
	Phosphorylcreatine	—	0·91	1·28

Release of creatine from phosphorylcreatine in the presence of purified diphosphopyridine nucleotide

The DPN sample used in the following experiments was purified by chromatography on Dowex 1 (formate). Analysis of the effluent indicated that it contained nicotinamide in an amount equivalent to 6% of the DPN. If it is assumed that nicotinamide arises as a result of the breakdown of DPN to ADP ribose, it may be concluded that the non-purified DPN is contaminated by 6% of ADP ribose. The purified DPN was also capable of bringing about the release of creatine from phosphorylcreatine at pH 7·4, but the amount of creatine released was less than that from an equivalent amount of the less pure commercial product (Table 12); the amount by which it was less was equivalent to twice the ADP ribose content of the less pure sample. It is clear then that some of the creatine released from phosphorylcreatine by the addition of commercial DPN is due to the presence of ADP ribose in the latter.

Table 12. *Release of creatine from phosphorylcreatine in the presence of purified and commercial diphosphopyridine nucleotide*

Reaction mixtures contained: ammediol buffer (pH 8·5), 100 μmoles; Mg^{2+} ion, 5 μmoles; phosphorylcreatine, 5 μmoles; enzyme, 7 mg. of Fraction 2; 2·5 μmoles of DPN. Total volume, 1·0 ml. Creatine release was determined after 4 hr. at 38°.

Nucleotide added	Creatine released (μmole)
Sigma DPN (purity, 95%)*	0·74
Purified DPN	0·45

* 2·5 μmoles of this sample was shown to contain 0·15 μmole of ADP ribose.

The activity of ADP ribose in bringing about the release of creatine from phosphorylcreatine suggested that the activity obtained with purified DPN may be due to the non-enzymic breakdown of the latter. Such a breakdown would not have been detected in previous assays, as comparisons were made with a standard DPN solution that had been incubated under the same conditions but in the absence of enzyme (cf. Table 8). Analysis of DPN at zero time and after incubation for 4 hr. (Table 13) showed that at pH 7·4 non-enzymic hydrolysis of DPN could not be detected. However, at pH 8·5 there was approximately 4–5% of hydrolysis. It would appear that the increased formation of AMP, ATP and creatine at pH 8·5 over that at pH 7·4 is due to the non-enzymic hydrolysis of DPN to ADP ribose. The lack of stoicheiometry at pH 7·4 between the amount of creatine released and the amount of ATP formed is presumably due to the limitations of the methods of assay.

The formation of AMP at pH 7·4 in the presence of the enzyme preparation, without detectable breakdown of DPN, could be accounted for by the relative insensitivity of the DPN assay, especially as analysis of the reaction mixture, by paper electrophoresis, after 4 hr. at pH 7·4 showed that nicotinamide (and therefore presumably ADP ribose) was formed from DPN. However, it is also possible that AMP may arise by enzymic hydrolysis at pH 7·4 of the α-isomer of DPN, which hydrolysis would not be detected by means of the cyanide and alcohol-dehydrogenase reactions. A check with purified *Neurospora* diphosphopyridine nucleotidase showed that the purified sample of DPN did contain 2·3% of the α-isomer and this could therefore make a contribution towards the amount of

Table 13. *Hydrolysis of diphosphopyridine nucleotide to adenosine 5′-phosphate and the conversion of adenosine 5′-phosphate into adenosine triphosphate in the presence of phosphorylcreatine*

Reaction mixtures contained: ammediol buffer, 100 μmoles; Mg^{2+} ion, 5 μmoles; DPN (purified), 2·5 μmoles; enzyme, 7·4 mg. of Fraction 2, which had been stirred at 2° with Nuchar C (50 mg./ml.); where indicated, phosphorylcreatine, 5 μmoles. Volume, 1·0 ml.; incubation time, 4 hr.; temp., 38°. For DPN assay 0·15 ml. samples were taken and the final volume was 3·6 ml.

Additions	DPN assay (enzymic method; $E_{340\,m\mu}$) Zero	4 hr.	Apparent DPN hydrolysis (μmole/ml.)	Creatine release (μmole/ml.)	AMP (μmole/ml.)	ATP (μmole/ml.)
pH 8·5						
None	0·710	0·680	0·106	0	0·246	0
Phosphorylcreatine	0·720	0·685	0·124	0·5	0	0·246
Boiled enzyme + phosphorylcreatine	0·720	0·692	0·099	0	0	0
pH 7·4						
None	0·729	0·726	<0·02	0	0·068	0
Phosphorylcreatine	0·708	0·704	<0·02	0·247	0	0·076
Boiled enzyme + phosphorylcreatine	0·710	0·713	<0·02	0	0	0

AMP formed. A third possibility is that even the purified DPN contains small amounts of ADP ribose. It has been found that whereas no ADP ribose could be detected by paper chromatography of freshly purified preparations of DPN, this compound could be detected after storage of the DPN either in solution or as a solid at 0° or −10°.

No detailed studies have been carried out with $DPNH_2$, but because this compound is more unstable than DPN it might be expected that non-purified samples would contain relatively larger amounts of ADP ribose. This could explain the greater activity of $DPNH_2$ over that of DPN in releasing creatine from phosphorylcreatine (Table 7) and inorganic phosphate from 1:3-diphosphoglycerate (Fig. 1). Chemical reduction of DPN may also give rise to ADP ribose as well as $DPNH_2$. If this were true, it would offer an explanation for the finding (Table 2) that purified DPN is active only after chemical reduction.

Release of creatine from phosphorylcreatine and formation of the hydroxamic acid derivative of 1:3-*diphosphoglycerate in the presence of adenosine diphosphate ribose and adenosine* 5′-*phosphate*

If the activity of the pyridine nucleotides is due to their non-enzymic conversion into, or contamination by, ADP ribose, which is then enzymically converted into AMP, it follows that similar results should be obtained when the pyridine nucleotides are replaced by AMP or ADP ribose. Indeed, this was found to be so. In the presence of 3-phosphoglycerate and the muscle enzyme, the addition of either of these compounds resulted in the formation of hydroxamic acids and, moreover, they were more effective than equivalent concentrations of DPN (Table 14). Higher concentrations of AMP inhibit the hydroxamic acid formation. The enzyme preparation was also capable of catalysing the overall reaction

AMP + 2 phosphorylcreatine → ATP + 2 creatine.

The equilibrium of the reaction was completely to the right, as the amount of creatine released was twice that of the added AMP.

Synthesis of phosphorylcreatine from 1:3-*diphosphoglycerate and* 3-*phosphoglycerate in the presence of oxidized or reduced diphosphopyridine nucleotide or adenosine* 5′-*phosphate*

Enzyme preparations from rat and rabbit skeletal muscle were shown to synthesize phosphorylcreatine under the conditions described by Cori *et al.* (1958) (Table 15). It was also possible to confirm Cori's observation that the synthesis of phosphorylcreatine was not decreased by the addition of an ATP-utilizing system in the form of the glucose–hexokinase system. It should be noted, however, that the reaction had ceased within 30 min. Kinetic studies were not attempted in view of the complexity of the system.

No tests have been made with 1:3-diphosphoglycerate but synthesis of phosphorylcreatine was achieved by the addition of either AMP or DPN to a system containing 3-phosphoglycerate and the muscle enzyme (Fig. 3).

Table 14. *Formation of hydroxamate from* 3-*phosphoglycerate in the presence of diphosphopyridine nucleotide, adenosine diphosphate ribose or adenosine* 5′-*phosphate*

Reaction mixtures contained: 3-phosphoglycerate, 8 μmoles; Mg^{2+} ion, 5 μmoles; ammediol buffer (pH 7·4), 100 μmoles; hydroxylamine (pH 7·0), 0·2 m-mole; enzyme, 7·4 mg. of Fraction 2, stirred for 15 min. at 2° with Nuchar C (50 mg./ml.); test nucleotide; volume, 1 ml. Tubes were incubated at 38° for 20 min. and hydroxamate formation was determined as in Table 4. The values have been corrected for 17% inhibition of the colour formation by the reaction mixture.

Nucleotide added	Concn. (mM)	Hydroxamate formation (μmoles/ml.)
DPN (purified)	2·5	0·474
	0·25	0·246
	0·10	0·043
ADP ribose	2·5	1·38
	0·25	0·474
	0·10	0·264
AMP	2·5	1·73
	0·25	3·32
	0·10	2·93
	0·05	2·57

Table 15. *Synthesis of phosphorylcreatine*

Reaction mixtures contained: phosphate buffer (pH 7·4), 25 μmoles; Mg^{2+} ion, 2·5 μmoles; cysteine (pH 7·4), 0·25 μmole; pyruvate, 2·5 μmoles; fructose 1:6-diphosphate, 1·25 μmoles; creatine, 5 μmoles; lactic dehydrogenase, 0·125 mg.; aldolase, 0·25 mg.; D-glyceraldehyde 3-phosphate dehydrogenase, 0·125 mg.; rabbit-muscle enzyme, 3·75 mg. of Fraction 2. Additions were: DPN, 0·5 μmole; glucose, 5 μmoles; hexokinase, 1 mg. All enzymes were stirred for 15 min. before use with Nuchar C (50 mg./ml.) and filtered. Volume, 0·55 ml.; temp., 38°.

Additions	Incubation time (min.)	Phosphorylcreatine synthesis (μmole/tube)
DPN (Sigma)	90	0·48
	180	0·50
DPN (purified)	90	0·43
DPN (purified) + glucose	30	0·41
DPN (purified) + glucose + hexokinase	30	0·43

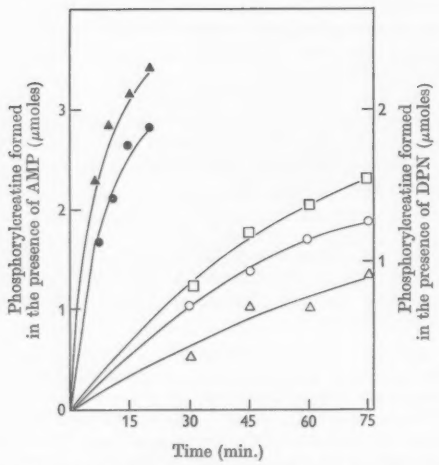

Fig. 3. Synthesis of phosphorylcreatine from creatine and 3-phosphoglycerate in the presence of adenosine 5′-phosphate or diphosphopyridine nucleotide. Reaction mixtures contained: phosphate buffer (pH 7·4), 50 μmoles; Mg^{2+} ion, 5 μmoles; creatine, 5 μmoles; 3-phosphoglycerate, 20 μmoles; AMP or DPN; enzyme, 7·4 mg. of Fraction 2 which had been stirred for 15 min. at 2° with Nuchar C (50 mg./ml.) and filtered. ▲, AMP, 0·25 μmole; ●, AMP, 0·1 μmole; □, DPN, 1·0 μmole; ○, DPN, 0·25 μmole; △, DPN, 0·1 μmole. Volume, 1 ml.; temp., 38°.

DISCUSSION

The results reported in this paper clearly indicate that reaction (1) described by Cori *et al.* (1958) and catalysed by extracts of rabbit and rat skeletal muscle cannot be regarded as a direct phosphoryl-group transfer, catalysed by a single enzyme. The evidence for this is twofold. Fractionation of the extracts results in a loss of activity, which is restored by recombination of two or more fractions, and no hydroxamic acid derivative of 1:3-diphosphoglycerate is formed on incubation of 3-phosphoglycerate and phosphorylcreatine with hydroxylamine and the enzyme. The overall reaction does occur in both directions when either DPN or $DPNH_2$ is present.

Presumably it is because the pyridine nucleotides were added as an essential part of the test systems that Cori *et al.* (1958) were able to obtain these reactions. DPN (and probably $DPNH_2$) does not appear to function as an intact molecule, but rather by virtue of its non-enzymic hydrolysis to ADP ribose (or as a result of its contamination by this compound), which in turn is enzymically converted into AMP. The experimental results indicate that AMP is converted into ATP, presumably via ADP, in the presence of either phosphoenolpyruvate or phosphorylcreatine. 1:3-Diphosphoglycerate may be involved directly in the conversion of AMP into ATP but, in any event, this compound would be converted non-enzymically into 3-phosphoglycerate, which would give rise to phosphoenolpyruvate. As ATP is formed and the appropriate glycolytic enzymes are present in the enzyme preparation, the synthesis of phosphorylcreatine from creatine and 1:3-diphosphoglycerate and the degradation of phosphorylcreatine in the presence of 3-phosphoglycerate can be accounted for by well-established reactions. Only catalytic amounts of adenine nucleotide need be formed because of the nature of the reactions.

It would seem that the failure of Cori *et al.* (1958) to obtain this overall reaction with heart-muscle preparations might well be ascribed to the absence of one or more of the enzymes required. Some preliminary experiments have shown that ATP is formed as a result of incubation of DPN (or $DPNH_2$) in the presence of phosphorylcreatine and partially purified preparations from rabbit- or pig-heart muscle, but no studies have been made of the overall reaction. It is also surprising that these authors failed to obtain phosphorylcreatine synthesis when DPN and 3-phosphoglycerate were incubated with a rat skeletal-muscle preparation.

The inability of Cori *et al.* (1958) to detect the presence of ADP and ATP in their incubation mixtures can be accounted for by the insensitivity of their assay procedure. The minimum amount of ADP (or ATP) which could be estimated by the method used was 0·005 μmole. As the sample taken for estimation was equivalent to 0·3 ml. of the reaction mixture, then the presence of ADP (or ATP) at a concentration of 17 μM would escape detection. Such a concentration of ADP was shown by these authors (Table 5 of their paper) to be effective in increasing the synthesis of phosphorylcreatine from creatine. It might also be mentioned that the values quoted by Cori *et al.* (1958) for the creatine-phosphoryltransferase activity of the muscle extracts are very low and in a number of experiments the values given for the amount of phosphorylcreatine synthesized in a 20 min. incubation period are taken to represent the rate of synthesis; they should, however, be regarded as the amount of phosphorylcreatine formed at equilibrium.

Strong evidence in favour of the non-participation of ADP and ATP was the finding by Cori *et al.* (1958) that whereas the synthesis of phosphorylcreatine from creatine and ATP was inhibited by the addition of glucose and hexokinase, that from creatine and 1:3-diphosphoglycerate was not. Because of the lack of kinetic studies, the interpretation of the results obtained is open to criticism.

There are no grounds for assuming that the affinity of hexokinase for ATP is greater than that of creatine phosphoryltransferase. Indeed, if the reverse were true then with high concentrations of creatine phosphoryltransferase (and very large amounts of this enzyme are present in extracts of skeletal muscle), no inhibition of the synthesis of phosphorylcreatine would be obtained. It would seem that the supposed inhibition of the creatine-phosphoryltransferase activity of the extracts could be explained as being due to the displacement of the equilibrium of the creatine-phosphoryltransferase reaction by the addition of glucose and hexokinase.

The conversion of AMP into ATP in the presence of either phosphoenolpyruvate or phosphorylcreatine implies that an enzyme(s) is present in the muscle extract which can convert AMP into ADP. Experiments with charcoal-treated preparations have shown that myokinase plus creatine phosphoryltransferase catalyse the overall reaction:

$$\text{AMP} + 2 \text{ phosphorylcreatine} \rightarrow \text{ATP} + 2 \text{ creatine}$$

although either enzyme alone is without effect. In this connexion it is of interest that pyruvic kinase catalyses the conversion of AMP into ATP in the presence of phosphoenolpyruvate when the enzyme is contaminated with myokinase (Bücher & Pfleiderer, 1955). Similar results were obtained by Chappell & Perry (1954) with creatine phosphoryltransferase, myokinase and phosphorylcreatine and confirmation of both these reactions has been reported by Molnar & Lorand (1960).

The hydrolysis of ADP ribose by the muscle preparation, and its failure to hydrolyse either DPN or $DPNH_2$, suggests that there may well be an enzyme which specifically hydrolyses ADP ribose. Jacobson & Kaplan (1957) have shown that extracts of pigeon liver contain a pyrophosphatase which hydrolyses ADP ribose but this enzyme also hydrolyses $DPNH_2$ although not DPN. A specific ADP ribose pyrophosphatase would provide a means of determining the contamination of both DPN and $DPNH_2$ samples by small amounts of ADP ribose.

SUMMARY

1. The reaction

$$\text{3-phosphoglycerate} + \textit{N}\text{-phosphorylcreatine} \uparrow\downarrow \text{1:3-diphosphoglycerate} + \text{creatine}$$

does not involve a direct phosphoryl-group transfer catalysed by a single enzyme.

2. The overall reaction in both directions is catalysed by preparations from rat and rabbit skeletal muscle in the presence of either oxidized or reduced diphosphopyridine nucleotide.

3. The pyridine nucleotides function by virtue of their non-enzymic conversion into, or as a result of their contamination by, adenosine diphosphate ribose, which undergoes enzymic hydrolysis to adenosine 5′-phosphate.

4. Skeletal-muscle preparations, treated with charcoal to remove adenine di- and tri-phosphate, catalyse the conversion of highly purified samples of adenosine 5′-phosphate into adenosine triphosphate in the presence of either *N*-phosphorylcreatine or phosphoenolpyruvate. Adenosine triphosphate is also formed when adenosine 5′-phosphate and *N*-phosphorylcreatine are incubated with myokinase and creatine phosphoryltransferase.

5. Rabbit skeletal muscle contains an enzyme which hydrolyses adenosine diphosphate ribose to adenosine 5′-phosphate, but which does not hydrolyse either oxidized or reduced diphosphopyridine nucleotide.

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Tricarboxylic Acid-Cycle Activity in *Streptomyces olivaceus*

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The tricarboxylic acid cycle occurs in mammals, plants and micro-organisms, although there is evidence indicating the occurrence of alternative pathways for terminal respiration (Krebs, 1954; Seaman & Naschke, 1955; Katagiri & Tochikura, 1958; Kornberg & Sadler, 1960). Amongst the genus *Streptomyces* this cycle appears to operate in *S. coelicolor* (Cochrane & Peck, 1953), *S. griseus* (Gilmour, Butterworth, Noble & Wang, 1955) and *S. nitrificans* (Schatz, Mohan & Trelawny, 1955). Maitra & Roy (1959*a*) have shown that *S. olivaceus* utilizes both the pentose phosphate pathway and the glycolytic route for the catabolism of glucose to the stage of pyruvic acid. The present paper is concerned with the metabolism of pyruvic acid through the tricarboxylic acid cycle and related processes.

MATERIALS AND METHODS

Chemicals. Diphosphopyridine nucleotide (DPN^+), triphosphopyridine nucleotide (TPN^+), reduced diphosphopyridine nucleotide (DPNH), reduced triphosphopyridine nucleotide (TPNH), flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD), horse-heart cytochrome *c* (mostly oxidized), silver–barium salt of phosphoenolpyruvic acid, sodium glyoxylate, phenazine methosulphate and crystalline bovine albumin were products of Sigma Chemical Co., St Louis, Mo., U.S.A.; reduced glutathione (GSH) and the disodium salt of adenosine triphosphate (ATP) were obtained from Schwarz Laboratories Inc., New York; DL-α-lipoic acid, *cis*-aconitic acid and DL(+)-*allo*-*iso*citric acid from California Corporation for Biochemical Research, Calif., U.S.A.; L_s(+)-*iso*citric acid was a generous gift from Dr H. A. Lardy; except where otherwise stated, the commercial variety of *iso*citric acid was used. Thiamine pyrophosphate was a gift from F. Hoffmann–La Roche and Co. Ltd., Basle, and antimycin A from Kyowa Fermentation Ind. Co., Tokyo. α-Oxoglutaric acid was a product of Fluka A.-G., West Germany, and oxaloacetic acid was obtained from Nutritional Biochemicals Corp., Ohio, U.S.A. Coenzyme A (CoA) was obtained from Pabst Laboratories, Milwaukee, U.S.A., and sodium fluoroacetate from L. Light and Co. Ltd. All ^{14}C-labelled compounds were from The Radiochemical Centre, Amersham, Bucks. Reduced cytochrome *c* was prepared either by reduction with a minimal amount of sodium dithionite and removal of the excess of reductant

by aeration for 10 min. at 0° or according to Margoliash (1954) by reduction with ascorbic acid, followed by adsorption on Decalso F (NH_4^+), elution with dilute ammonia, dialysis and freeze-drying. 2:4-Dinitrophenol was recrystallized from water; *p*-phenylenediamine hydrochloride was likewise recrystallized twice just before use.

Microbiological procedures. The maintenance of *S. olivaceus* NRRL B-1125, the preparation of the inoculum culture and the growth of the organism in the complex medium have been described (Maitra & Roy, 1959*a*), except that a reciprocating shaker (200 strokes/min.) was used at room temperature (approx. 25°) for growing the organism. Such cells are referred to as glucose-grown cells. For the manometric experiments starved cells (Maitra & Roy, 1959*a*) were used. For cell-free extracts cells were harvested at the end of the 24 hr. growth and washed thrice with potassium chloride solution (1·15%) by centrifuging in the cold; the packed cells so obtained could be kept at −20° for a fortnight without an appreciable decrease in *iso*citric-dehydrogenase activity. Cell-free extracts were prepared by disrupting, in a mechanical shaker (Nossal, 1953), 6 g. of thawed cells with 10 g. of acid-washed glass beads (Superbrite, type 133; Minneapolis Mining and Manufacturing Co., St Paul, Minnesota) and 8 ml. of 0·1 M-potassium phosphate buffer, pH 7·2. Four shakes, each of 30 sec. duration, were applied and the cartridge was cooled in ice between shakings. The yellowish, opalescent supernatant solution obtained after centrifuging at 18 000*g* for 30 min. was used as the crude source of enzymes. In some experiments the 18 000*g* particulate preparation was obtained by a preliminary clarification of the extract for 20 min. at 2000*g* to remove the intact cells, debris and glass beads, followed by centrifuging at 18 000*g* for 30 min.; the reddish sediment was suspended in 2 ml. of 0·1 M-potassium phosphate buffer, pH 7·2. The growth rate of *S. olivaceus* was very slow in a synthetic medium (Maitra & Roy, 1960) containing acetate as the sole source of carbon, and this could not be improved even after a number of transfers through such media containing decreasing proportions of glucose and increasing proportions of acetate. Cells were grown in the medium of Maitra & Roy (1960) with sodium acetate (1 g./100 ml.) replacing glucose, and containing Difco yeast extract (25 mg./100 ml.). The acetate-grown cells were harvested after 72 hr. of growth and cell-free extracts were prepared immediately as described for glucose-grown cells. Such cells were very resistant to rupture and the cell-free extracts usually contained only one-fifth of the protein of those obtained from glucose-grown cells. Experiments, unless otherwise stated, were with extracts from glucose-grown cells.

*Enzyme assays. iso*Citric dehydrogenase was estimated by TPN^+ reduction at 340 mμ in the presence of *iso*citrate (Ochoa, 1955*a*); aconitase by TPN^+ reduction in the presence of citrate and also *cis*-aconitate. The increase of extinction at 240 mμ after addition of *iso*citrate and citrate was also used to measure aconitase activity (Racker, 1950); in some experiments the reaction rate was also measured by following the decrease in extinction at 240 mμ due to *cis*-aconitate after the addition of TPN^+. Succinic dehydrogenase was estimated by the manometric method outlined by Singer & Kearney (1957), with phenazine methosulphate, and in some experiments also by the method of Ells (1959) by coupling the above-mentioned system with 2:6-dichlorophenol-indophenol and measuring the reduction of the latter at 600 mμ. Malic dehydrogenase was measured at 340 mμ by DPNH or TPNH oxidation with oxaloacetate (Ochoa, 1955*b*) and glutamic and α-alanine dehydrogenases by the oxidation of reduced pyridine nucleotide at 340 mμ in the presence of NH_4^+ ion and the corresponding keto acids (Strecker, 1955). Oxidases for the reduced pyridine nucleotides were assayed by measuring the decrease in extinction at 340 mμ in the presence of TPNH and DPNH (Dolin, 1959). TPNH–cytochrome *c* reductase and DPNH–cytochrome *c* reductase were measured at 500 mμ by following the rate of reduction of the oxidized cytochrome *c* in the presence of TPNH or DPNH respectively (Horecker, 1955; Mahler, 1955*a*). Transhydrogenase was assayed by the method outlined by Hochster & Katznelson (1958), which is based on the fact that preparations with greater DPNH-oxidase activity than TPNH oxidase would show higher oxidation rate of reduced pyridine nucleotide on addition of DPN^+ to a cuvette in which a steady rate of TPNH oxidation has already ensued. Fumarase was tested spectrophotometrically (Racker, 1950) at 240 mμ with both malate and fumarate as the substrate. Flavins were determined according to Strittmatter (1959). Lactic-dehydrogenase activity was measured according to Mehler, Kornberg, Grisolia & Ochoa (1948). Diaphorase activity was measured by the reduction of 2:6-dichlorophenol-indophenol in the presence of cyanide with TPNH or DPNH as substrate (Mahler, 1955*b*). *iso*Citratase was estimated spectrophotometrically by the rate of change of extinction at 252 mμ due to the formation of glyoxylate semicarbazone, according to Olson (1959). Incubation mixtures for detecting the formation of citrate from acetate and oxaloacetate and of malate from acetate and glyoxylate were similar to those of Kornberg & Madsen (1958) and contained the following components (μmoles): potassium phosphate buffer, pH 7·2, 100; magnesium chloride, 10; ATP, 10; GSH, 4; CoA, 0·1; [1-^{14}C]acetate, 5(2 μc/mole); oxaloacetate or glyoxylate, 10; enzyme solution containing 0·5 and 2·0 mg. of protein for extracts from glucose-grown and acetate-grown cells respectively. The total reaction volume was 0·7 ml. and 1·5 ml. respectively with extracts from glucose- and acetate-grown cells. Incubation was at 37° for 1 hr. in air. Reaction products were detected by radioautography. The synthesis of radioactive malate from glyoxylate and labelled acetate was taken as an indication of malate-synthetase activity (Kornberg & Madsen, 1958) and of labelled citrate from oxaloacetate and [2-^{14}C]acetate as condensing-enzyme activity (Ochoa, Stern & Schneider, 1951). In all cases the wavelength scale of the spectrophotometer was checked beforehand by reference to the isosbestic point of bromocresol green (Parthasarathy & Sanghi, 1958).

Preparation and measurement of radioactive samples. Cell-free extracts after incubation were deproteinized, where necessary, by warming for 2 min. at 50° with twice the volume of 80% ethanol, the precipitated proteins were washed twice with it by centrifuging at 2000*g* and the combined supernatant solutions were kept at 0° over pellets of sodium hydroxide until dry. Chromatography was carried out at room temperature on Whatman no. 1 papers by the ascending method, with butan-1-ol–acetic acid–water (4:1:1, by vol.) containing sodium acetate (0·05 g./100 ml. of the irrigating solvent) to prevent the formation of acid bands on the developed chromatogram

(Lawson & Hartley, 1958), and after the solvents were removed by air-drying the paper was sprayed with bromocresol green in ethanol (0·04%, w/v), adjusted to pH 7; the carboxylic acids appeared as yellow spots on a blue background. Radioautography and subsequent radioassay were carried out according to Kornberg (1958), with Ilford X-ray films with a contact period of 3 weeks. Identification of compounds on chromatograms was always made by comparison with authentic samples. Incubations with sodium [^{14}C]bicarbonate were carried out with cells grown for 72 hr. in the synthetic medium (Maitra & Roy, 1960) containing glucose or acetate; washed cells were suspended in fresh growth medium buffered strongly at pH 7·6 and containing sodium [^{14}C]bicarbonate (10 μc/ml., specific activity 1 mc/m-mole) and were incubated aerobically on the reciprocating shaker at room temperature. After incubation cells were collected by centrifuging, washed twice with cold sodium bicarbonate (1%, w/v) and treated thrice with hot 80% ethanol (Kornberg, 1958) to extract the tricarboxylic acid-cycle intermediates. The cell residue was analysed for amino acids by paper chromatography after acid hydrolysis (Maitra & Roy, 1959*b*). All other methods of radioassay on planchets and on paper were as described previously (Maitra & Roy, 1959*a*).

Analytical procedures. α-Keto acids were determined as 2:4-dinitrophenylhydrazones by the procedure of Friedemann & Haugen (1943). Other methods were as described by Maitra & Roy (1959*a*).

RESULTS

Experiments with whole cells

The ability of intact cells to oxidize the tricarboxylic acid-cycle intermediates and related compounds was examined with suspensions of non-proliferating cells harvested during the early logarithmic phase of growth. In view of the permeability barriers of the microbial cell wall to some of these compounds (Ajl, 1958), experiments were also conducted at a lower pH. The rate of oxidation of different substrates varied considerably from preparation to preparation: for instance, Q_{O_2} (N) for succinate after subtraction of the endogenous values were in the range 34·2 to 67·3. The relative rates were more or less the same. Typical data are shown in Table 1, which indicate that practically all the intermediates are oxidized, assuming that the endogenous oxidation rate is unaffected in the presence of the substrates. The oxidation of all these substrates except glucose ensued after a small initial lag and continued steadily during the next 5 hr. period. The greater rate of oxygen uptake with pyruvate in the presence of a relatively small amount of fumarate, compared with that observed with either substrate alone, suggests the catalytic role of at least one of the intermediates of the cycle. The respiration rates with succinate, oxaloacetate, malate and *iso*citrate are increased at the lower pH. The rate of acetate oxidation was greater with cells grown on this substrate; Q_{O_2} (N) (after subtraction of the endogenous control value) for an acetate-grown organism was 61·2, whereas that for glucose-grown cells was 30·1 under conditions shown in Table 1.

Isotopic carbon (^{14}C) incorporated into carbon dioxide from [1-^{14}C]- and [2-^{14}C]-acetate by the non-proliferating cells was respectively 34·1 and 5·6%.

Experiments with cell-free extracts

To avoid the permeability effects with the intact cells and to lower the endogenous metabolism of *S. olivaceus*, subsequent experiments were mostly carried out with cell-free systems. The capacity of such preparations to metabolize the intermediates of the citric acid cycle was tested with malate and succinate (Table 2).

Enzyme assays. Before estimating the pyridine nucleotide-linked reactions in extracts of *S. olivaceus*, the oxidases for DPNH and TPNH and transhydrogenase were assayed in order to avoid ambiguity in data relating to pyridine nucleotide specificity. The cell-free extracts possess a low DPNH-

Table 1. *Rates of oxidation of tricarboxylic acid-cycle intermediates by resting cells of* Streptomyces olivaceus

Cells were harvested at the end of growth for 24 hr. The complete system contained, in a total volume of 3·0 ml.: starved cells equivalent to 0·45 mg. of nitrogen in 200 μmoles of potassium phosphate buffer, pH 7·2, and neutralized substrate, 50 μmoles (except where otherwise stated), in the side arm. The centre well contained 0·2 ml. of 20% (w/v) potassium hydroxide on filter paper. Temperature was 37°, gas phase air and shaker speed 120 strokes/min. Preincubation for 10 min. was allowed before the contents of the side arm were tipped in. The specific rate of oxygen uptake is expressed as Q_{O_2} (N) (μl. of oxygen consumed/mg. of cell nitrogen/hr.), based on the reading at 60 min. Values for oxaloacetate, *cis*-aconitate and fumarate have been corrected for blanks in the absence of the cell suspension.

Substrate	Q_{O_2} (N) pH 7·2	Q_{O_2} (N) pH 5·5
None	201·1	54·0
D-Glucose	251·1	21·6
Pyruvate	232·5	76·8
Acetate	219·2	40·3
Oxaloacetate	260·1	281·8
Citrate	218·2	64·3
cis-Aconitate	406·3	134·5
*iso*Citrate	212·0	84·0
Glyoxylate	220·7	—
α-Oxoglutarate	281·3	45·9
Succinate	248·4	152·6
Fumarate	235·2	67·4
DL-Malate	285·8	368·2
Fumarate*	218·0	58·1
Pyruvate + fumarate†	373·6	103·1

* Fumarate 2 μmoles.
† Pyruvate 50 μmoles and fumarate 2 μmoles.

oxidase activity but neither TPNH oxidase nor transhydrogenase was detected (Fig. 1). The DPNH-oxidase activity varied from preparation to preparation (range 0·3–0·7, as measured by the change in extinction at 340 mμ/hr./mg. of protein). The enzyme solution lost about 80% of its DPNH-oxidase activity in 18 hr. on storage at −20°. The oxidase activity was insensitive to cyanide (mM) but was inhibited (84%) by antimycin A (0·01 mM).

The presence of a TPN^+-linked, Mg^{2+}-dependent *iso*citric dehydrogenase is indicated in Fig. 2; no DPN^+-linked activity could be detected under conditions of assay described by Kornberg (1955), or in preparations containing only negligible DPNH-oxidase activity after overnight storage at −20°, although the TPN^+-linked enzyme withstood storage at this temperature for 10 days without measurable loss of activity. The enzyme was not inhibited by mM-cyanide. The commercial variety of the substrate, namely DL(+)-*allo*-*iso*citric acid, elicited a quicker rate of reaction than did the natural enantiomorph. That the product of reaction was α-oxoglutaric acid was suggested by the chromatographic behaviour of the 2:4-dinitrophenylhydrazine derivative obtained from the incubated mixture (El Hawary & Thompson, 1953) and also by the absorption spectrum of these derivatives eluted from the chromatograms. The reversibility of the *iso*citric-dehydrogenase reaction could not be demonstrated by the oxidation of TPNH in a system containing bicarbonate, α-oxoglutarate and the enzyme solution.

The *iso*citric-dehydrogenase activity was used to measure aconitase by replacing *iso*citrate with

Table 2. *Oxidation of* DL-*malate and succinate by cell-free extracts of* Streptomyces olivaceus

The complete system for malate contained the following components (μmoles): magnesium chloride, 6; manganese sulphate, 6; potassium phosphate buffer, pH 7·2, 200; DPN^+, 0·5; TPN^+, 0·5; substrate, 50; phenazine methosulphate 5; enzyme solution equivalent to 5·5 mg. of protein. The system for succinate was the same except that DPN^+ and TPN^+ were omitted and enzyme solution used contained 9·0 mg. of protein. Details were as given in Table 1. Results are expressed as μl. of oxygen consumed in 40 min.

Reaction mixture	Uptake of O_2 (μl.) With malate	With succinate
Complete	138	149
Without $MgCl_2$	122	144
Without $MnSO_4$	131	142
Without $MgCl_2$ and $MnSO_4$	91	122
Without DPN^+	121	—
Without TPN^+	110	—
Without DPN^+ and TPN^+	88	—
Without phenazine methosulphate	61	13
Without DPN^+, TPN^+ and phenazine methosulphate	29	—
Without substrate	25	17

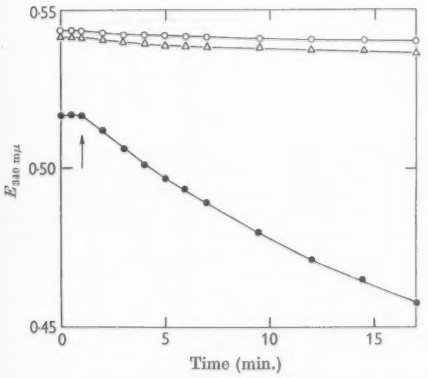

Fig. 1. Oxidation of reduced pyridine nucleotides by crude extracts of *S. olivaceus*. The reaction mixture contained (μmoles): potassium phosphate buffer, pH 7·2, 280; magnesium chloride, 10; reduced pyridine nucleotide, approx. 0·1; DPN^+, where indicated, 0·1; enzyme protein, 0·5 mg. in a total volume of 3·0 ml. Reactions were started with addition of the enzyme solution at the instant indicated by the arrow. ○, TPNH; △, TPNH + DPN^+; ●, DPNH.

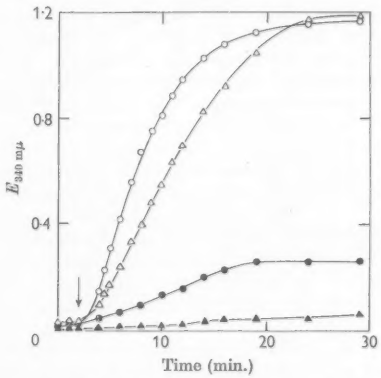

Fig. 2. *iso*Citric-dehydrogenase activity in cell extract of *S. olivaceus*. The reaction mixture contained, in a total volume of 3·0 ml., the following (μmoles): potassium phosphate buffer, pH 7·2, 265; magnesium chloride, 10; pyridine nucleotide, 0·2; substrate, 10; crude extracts, equivalent to 0·6 mg. of protein. The pair of cuvettes containing DPN^+ received 3 μmoles of AMP each. Additions and omissions are as indicated. Substrates were added at the instant marked by the arrow; the control cuvette contained water in place of the substrate. ○, *iso*Citrate + TPN^+; △, DL(+)-*iso*citrate + TPN^+; ●, *iso*citrate–Mg^{2+} + TPN^+; ▲, *iso*citrate + DPN^+.

citrate and *cis*-aconitate and measuring the reduction of TPN^+ (Fig. 3). A distinct lag was observed with citrate. Aconitase was also measured by following at 240 mμ the appearance of *cis*-aconitate from citrate and *iso*citrate or its disappearance. In the latter case the removal of *cis*-aconitate was favoured by the presence of TPN^+, which presumably shifted the equilibrium towards α-oxoglutarate. Unlike *iso*citric dehydrogenase, aconitase lost more than 60% of its activity on storage at −20° for 18 hr. Citratase, which splits citric acid into oxaloacetate and acetate (Katagiri & Tochikura, 1959), could not be detected in the extract by coupling oxaloacetate *in situ* to malic dehydrogenase under conditions shown in Fig. 6.

Oxidation of α-oxo acids, etc. Reduction of DPN^+ in the presence of pyruvate or α-oxoglutarate was not observed in any of the extracts supplemented with CoA, thiamine pyrophosphate, GSH, DL-α-lipoic acid, Mg^{2+} and Mn^{2+} ions (King, Kawasaki & Cheldelin, 1956), and the addition of 5′-adenylic acid (AMP) and also biotin (Cantino, 1951) had no effect; nor was TPN^+ reduced by these substrates.

Fig. 3. Aconitase in *S. olivaceus* extract. The reaction mixture contained (μmoles): potassium phosphate buffer, pH 7·2, 260; magnesium chloride, 10; pyridine nucleotide, 0·4; substrate, 10; enzyme solution equivalent to 0·35 mg. of protein. Other details were as given for Fig. 2. ●, *cis*-Aconitate + TPN^+; ○, citrate + TPN^+; △, citrate + DPN^+.

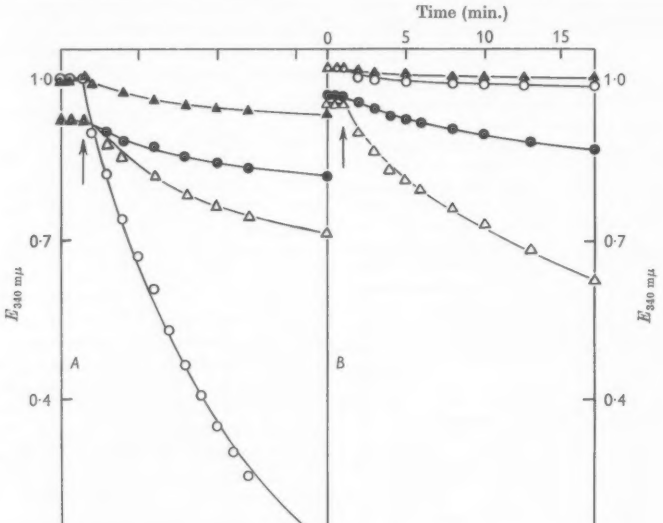

Fig. 4. Glutamic dehydrogenase (*A*) and alanine dehydrogenase (*B*) in *S. olivaceus* extract. The complete reaction mixture contained, in a total volume of 3·0 ml., the following (μmoles): potassium phosphate buffer, pH 7·2, 270; magnesium chloride, 10; dibasic ammonium phosphate, 10; α-oxoglutarate (αKG) or pyruvate (*A* and *B* respectively), 10; reduced pyridine nucleotide, approx. 0·2; enzyme solution equivalent to 0·5 mg. of protein. Arrows indicate addition of the enzyme. (*A*) ▲, αKG + TPNH; ●, αKG + DPNH; △, αKG + NH_4^+ + DPNH; ○, αKG + NH_4^+ + TPNH. (*B*) ▲, Pyruvate + TPNH; ●, pyruvate + DPNH; △, pyruvate + NH_4^+ + DPNH; ○, pyruvate + NH_4^+ + TPNH.

The coupling of DPN^+ reduction in the above-mentioned systems with cytochrome *c* was also ineffective (Imamoto, Iwasa & Okunuki, 1959). With GSH omitted from the above-mentioned system no reduction of 2:6-dichlorophenol-indophenol was observed (Imamoto *et al.* 1959) even in the presence of FMN or FAD. The absence of lactic dehydrogenase as tested by the lack of oxidation of DPNH with added pyruvate also eliminated a route whereby the pyruvic-dehydrogenase action could be masked. This was also confirmed by the fact that no disappearance of pyruvate or α-oxoglutarate occurred after 1 hr. incubation of these substrates with the system containing CoA, thiamine pyrophosphate, GSH, DPN^+, DL-α-lipoic acid, Mg^{2+} ion and the enzyme solution. In this regard no difference was observed between the 18 000*g* supernatant and the corresponding particulate preparation.

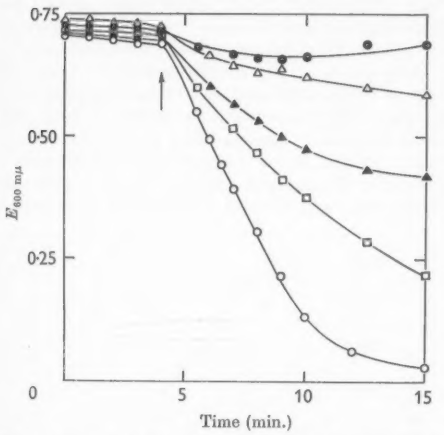

Fig. 5. Succinic-dehydrogenase activity in *S. olivaceus* cell extracts. The complete reaction mixture contained, in a total volume of 3·0 ml., the following (μmoles): potassium phosphate buffer, pH 7·2, 210; magnesium chloride, 10; potassium cyanide, 10; 2:6-dichlorophenol-indophenol, 0·04; phenazine methosulphate, 2·5; cell-free extract equivalent to 1·3 mg. of protein; succinate and malonate where indicated. ○, Succinate, 10 μmoles; □, succinate and malonate, 10 μmoles each; ▲, succinate, 10 μmoles, and malonate, 20 μmoles; △, succinate, 10 μmoles, and malonate, 30 μmoles; ●, malonate, 20 μmoles. In the last curve (●), almost the same changes of extinction were observed with 10 or 30 μmoles of malonate. Cuvettes containing all the additions except 2:6-dichlorophenol-indophenol were adjusted to 100% transmission. The reaction was started with phenazine methosulphate at the time indicated by the arrow. The results have been corrected for the reduction of 2:6-dichlorophenol-indophenol in the absence of the substrate and also for that in the absence of the enzyme solution.

The presence in the extract of an active glutamic dehydrogenase is shown in Fig. 4. The equilibrium of the reaction is far in favour of synthesis of glutamate, for no reduction of DPN^+ or TPN^+ was observed with glutamate. Similar observations were made with alanine dehydrogenase (Fig. 4), except that glutamic dehydrogenase was more active with TPNH, whereas the latter acted only with DPNH. The formation of glutamate and alanine was confirmed by chromatographic examination of the incubated mixtures.

Although succinate was oxidized by resting cells, very little oxidation took place when the cell-free extract was incubated with succinate unless phenazine methosulphate was present (Table 2); chromatography of the reaction product showed a little malate to have been produced and no fumarate. Fumarate, as measured by its extinction at 240 mμ (Racker, 1950), could not be detected as a product of succinate dehydrogenation during a 20 min. reaction period. No reduction of 2:6-dichlorophenol-indophenol or of oxidized cytochrome *c* was observed on addition of succinate to a system containing cyanide, enzyme and bovine albumin (Singer & Kearney, 1957) as well as FMN or FAD. Phenazine methosulphate, however, was effective as a mediator between 2:6-dichlorophenol-indophenol and possibly succinate, as measured by the rapid reduction of 2:6-dichlorophenol-indophenol under conditions shown in Fig. 5. Malonate, which was rapidly oxidized by intact cells, caused little reduction of the indophenol dye under these conditions. Increasing inhibitions were produced as the malonate:succinate ratio was increased (Fig. 5), although the inhibitory concentrations were higher than were usually employed (Pardee & Potter, 1949). The cell-free extracts were devoid of succinate-cleaving enzyme (Seaman & Naschke, 1955) as measured by the oxidation of DPNH in the presence of succinate, ATP and CoA.

Fumarase was not detected. Chromatographic experiments indicated that no tricarboxylic acid-cycle intermediate was formed in detectable amounts on incubating fumarate with the cell-free extracts.

Extracts of *S. olivaceus* contained an active DPNH-linked malic dehydrogenase (Fig. 6) and also a TPNH-linked enzyme, the latter being less active. The reaction product in each case was co-chromatographed with authentic malate. In the forward direction addition of malate did not result in reduction of DPN^+, although with TPN^+ slight reduction occurred (extinction change at 340 mμ/hr./mg. of enzyme protein, 0·36 under conditions described in Fig. 2). The possibility that this small reduction of TPN^+ might be due to malic enzyme (Ochoa, Mehler & Kornberg, 1948) could not be ruled out, although no oxidation of TPNH was

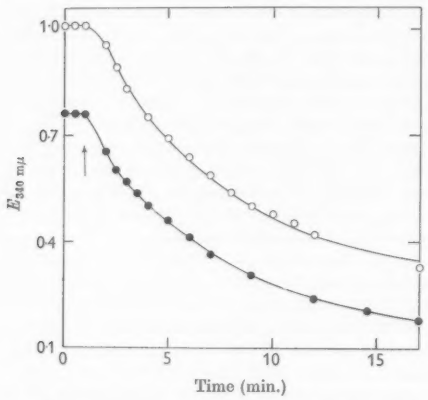

Fig. 6. Malic-dehydrogenase activity in crude extracts of *S. olivaceus*. The reaction mixtures contained the following components (μmoles): oxaloacetate, 5; reduced pyridine nucleotide, approx. 0·2; enzyme protein, 0·5 mg. and 8 μg. for the TPNH-linked and DPNH-linked reactions respectively. Other details were as given for Fig. 2. At the time denoted by the arrow reactions were started with the enzyme. There was no demonstrable activity for DPNH oxidase or TPNH oxidase at the concentration of enzyme solution used in these experiments. ○, Oxaloacetate + TPNH; ●, oxaloacetate + DPNH.

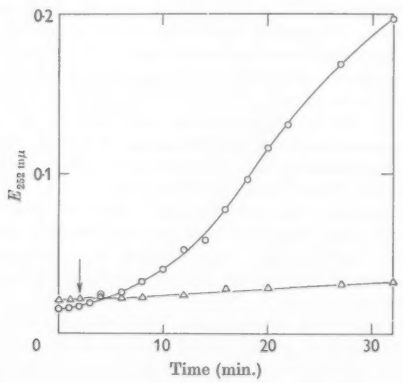

Fig. 7. *iso*Citratase in *S. olivaceus* extracts. The reaction mixture contained, in a total volume of 3·0 ml., the following (μmoles): potassium phosphate buffer, pH 6·0, 230; magnesium chloride, 10; GSH, 4; semicarbazide hydrochloride, neutralized to pH 6·0, 60; *iso*citrate, 10; enzyme solution equivalent to 0·18 and 0·13 mg. of protein for the acetate-grown and the glucose-grown cells respectively. Reactions were started with the addition of *iso*citrate at the time indicated by the arrow. Controls lacked the substrate. ○, Acetate-grown cells; △, glucose-grown cells.

observed in a system composed of pyruvate, Mg^{2+} and Mn^{2+} ions, bicarbonate, phosphate and the enzyme.

Recent work on the synthesis of cell constituents from C_2 units has led to the formulation of the glyoxylate cycle as a variant of the tricarboxylic acid cycle, particularly in acetate-grown micro-organisms (Kornberg & Krebs, 1957). The two key enzymes of this cycle, *iso*citratase and malate synthetase, were sought in *S. olivaceus*. Extracts from cells grown on glucose contained no *iso*citratase when assayed by the procedure outlined in Fig. 7, nor was the 2:4-derivative of glyoxylate detected by chromatography of the reaction mixture. Malate synthetase, measured as described in the Materials and Methods section, was also not detected. The acetate-grown cells, however, contained both *iso*citratase (Fig. 7) and malate synthetase, the latter being indicated by the observation that malate was the only labelled compound formed from [^{14}C]acetate and glyoxylate. Both the glucose-grown and the acetate-grown cells formed [^{14}C]citrate from oxaloacetate and labelled acetate.

Components of the electron-transport chain. In view of the general aerobic nature of organisms of the genus *Streptomyces* the occurrence in such cells of an electron-transport chain to oxygen is probable. Heim, Silver & Birk (1957) examined a number of *Streptomyces* species for their cytochrome composition and showed cytochromes of groups *b*

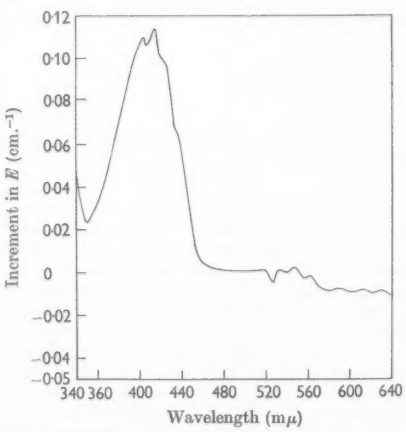

Fig. 8. Difference spectrum (reduced minus oxidized state of pigments) of crude extracts of *S. olivaceus*. The reaction mixture contained the following (μmoles): potassium phosphate buffer, pH 7·2, 270; potassium ferricyanide (used for oxidation of pigments), 300; sodium dithionite (used for reduction of pigments), 400; crude extract equivalent to 6·0 mg. and 2·1 mg. of protein for work above 380 mμ and below 380 mμ respectively.

and *c* to be present. The cell-free extracts of *S. olivaceus* were tested chromatographically for flavins: two spots corresponding respectively to riboflavin and FMN were detected, but no FAD. To determine the absorption peaks of the respiratory components extracts (5–20 mg. of protein) were scanned on the Beckman spectrophotometer model DU in the range 330–700 mμ in the presence of potassium ferricyanide (0·3 mM) and sodium dithionite (4 mM) for oxidized and reduced states respectively; dilute extracts were used for work below 380 mμ. It was assumed that scattering of light by the opalescent extracts was independent of the state of oxidation of the pigments (Ghiretti-Magaldi, Giuditta & Ghiretti, 1958), and the difference spectra were plotted (Fig. 8) in terms of the difference in extinction between the reduced and the oxidized pigments ($A_{red.} - A_{ox.}$, where A terms denote extinction). They were found to be qualitatively the same in every sample of crude extract. The following absorption maxima (mμ) were noted: 340, 405, 417, 520, 532, 546, 551–553, 562, 590, 610 and 630. The peak in the Soret region at 417 mμ was the most prominent and that in the region 551–553 mμ was the least sharp.

The cell-free extracts of *S. olivaceus* failed to bring about oxidation of reduced cytochrome *c*, as measured by the decrease in extinction at 550 mμ after the addition of the enzyme solution to a mixture containing reduced cytochrome *c*, potassium phosphate buffer, pH 7·2, and Mg^{2+} or Mn^{2+} ions. Nor was oxidation observed with the particulate preparation (18 000*g*). This inertness of the cell-free preparations of *S. olivaceus* towards re-

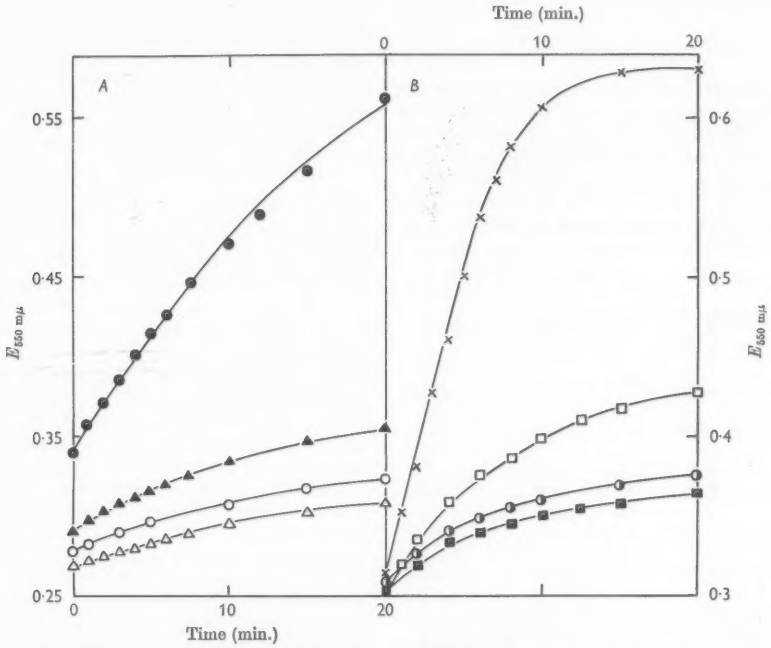

Fig. 9. TPNH-cytochrome *c*-reductase (*A*) and DPNH-cytochrome *c*-reductase (*B*) activity in cell-free extracts of *S. olivaceus*. The complete reaction mixture contained: potassium phosphate buffer, pH 7·2, 260 μmoles; magnesium chloride, 10 μmoles; DPNH or TPNH, 0·2 μmole; FAD or FMN, 0·1 μmole; oxidized cytochrome *c*, 25 μm-mole; enzyme solution equivalent to 0·7 mg. of protein; total volume, 3·0 ml. Controls were without the flavin nucleotides and were run separately with each assay. Cuvettes containing all additions except cytochrome *c* were adjusted to 100% transmission. Reactions were started with addition of the enzyme solution after the readings were steady for at least 2 min. The extinction readings for the complete system with FMN (*B*) have been corrected for the non-enzymic reduction of cytochrome *c* in the presence of FMN and DPNH. During the first 3 min. this amounted to 7% of the extinction changes obtained in the complete system. No such reduction of cytochrome *c*, however, took place with FMN and TPNH, FAD and DPNH or with FAD and TPNH. (*A*) ●, FMN; ○, control; ▲, FAD; △, control. (*B*) ×, FMN; ◑, control; □, FAD; ■, control.

duced cytochrome *c* was paralleled by the observation that whole resting cells of this organism also failed to oxidize *p*-phenylenediamine (0·02 M) (Richardson, 1957) under conditions described in Table 1.

The fresh extracts exhibited TPNH–cytochrome *c*-reductase and DPNH–cytochrome *c*-reductase activity. Thus added ferricytochrome *c* was slowly reduced in the presence of the extracts by TPNH or DPNH (Fig. 9). The addition of FMN stimulated both enzyme activities though FAD was less active. Fig. 10 shows the presence in the crude extracts of diaphorase activity. An increase in activity of approximately 20% was observed on omitting cyanide from the assay system. No attempt was, however, made to ascertain the extent to which the cytochrome *c*-reductase activities were responsible for the observed reduction of the dye by TPNH and DPNH (Mahler, 1955*b*).

DISCUSSION

In the present work some evidence has been put forward for the operation of the tricarboxylic acid cycle in *S. olivaceus*, although the oxidative decarboxylation of α-oxo acids and fumarase activity could not be demonstrated in the cell-free extract. This might be due to the loss of activity of the relevant enzyme systems during preparation.

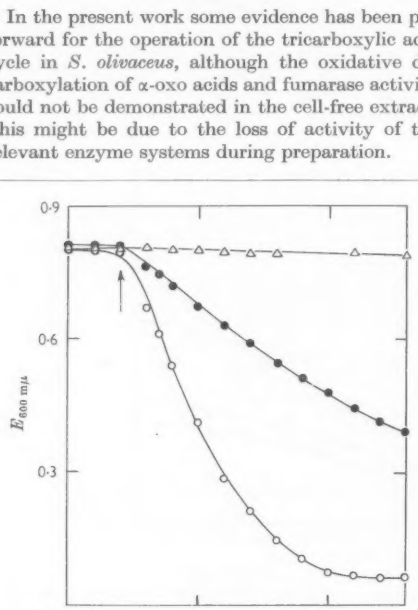

Fig. 10. Reduction of 2:6-dichlorophenol-indophenol by extracts of *S. olivaceus*. The reaction mixture contained: potassium cyanide, 10 μmoles; 2:6-dichlorophenol-indophenol, 0·05 μmole; enzyme protein, 0·4 mg. Other details were as given for Fig. 1. The control cuvette contained all additions except the dye solution and the substrate. △, No addition; ● TPNH; ○, DPNH.

The cell-free extract from *S. olivaceus* is capable of converting acetate into citrate in the presence of oxaloacetate, CoA, ATP and inorganic phosphate; the formation of citrate may be due to the joint action of acetokinase and phosphotransacetylase, or acetyl-CoA kinase (Berg, 1956) and the condensing enzyme. Citrate in its turn is isomerized to *iso*citrate by aconitase with intermediate formation of *cis*-aconitic acid; in the presence of TPN^+ all the three intermediates give rise to α-oxoglutaric acid.

α-Oxo acids caused considerable oxygen uptake over the endogenous respiration of whole cells (Table 1) and the involvement of a typical keto acid-oxidase system, as suggested by the inhibition of oxidation by arsenite (Mehler, 1957). The cell-free extract, however, did not oxidize these substrates. Possibly enzyme components were damaged irreversibly or depleted of some unknown cofactors. The absence in the cell-free extracts of detectable levels of fumarase is also not consistent with data from whole cells, where fumarate exerts some catalytic action on the oxidation of pyruvate (Table 1). The balance of evidence, however, is in favour of the operation of tricarboxylic acid cycle in *S. olivaceus*, but how far this is utilized in the economy of the cell remains to be determined.

Growth of *S. olivaceus* on acetate is associated with the appearance in the mycelia of *iso*citratase and malate synthetase. In the acetate-grown cells therefore acetyl-CoA has two separate points of entry into the tricarboxylic acid cycle, namely, through condensing enzyme producing citrate and through malate synthetase giving malate. According to Kornberg (1958), such a situation would allow the cell to utilize for its synthetic reactions carbon skeletons of tricarboxylic acid-cycle intermediates, in addition to those contributed by the carbon dioxide-fixation reactions that have been shown to occur in both the acetate-grown and the glucose-grown organisms.

The DPNH-oxidase system of *S. olivaceus* could explain only a fraction of oxygen consumption of whole cells, and neither TPNH oxidase nor transhydrogenase could be detected. The regeneration of TPN might be linked with processes such as the reduction of oxaloacetate (Fig. 6), reductive amination of α-oxoglutarate (Fig. 4) or those catalysed by glutathione reductase or similar enzymes. The inability of succinate to reduce cytochrome *c* distinguishes the electron-transport system of this substrate from those of TPNH and DPNH (Slater, 1958). Succinate dehydrogenation was achieved only when the artificial carriers phenazine methosulphate and 2:6-dichlorophenol-indophenol were present. Eichel (1956) considers that in *Tetrahymena*, which is devoid of cytochrome *c* oxidase, the role of cytochrome *c* in DPNH–cytochrome *c* reductase is that of an artificial carrier. In *S.*

olivaceus attempts to demonstrate cytochrome *c* oxidase with mammalian cytochrome *c* were unsuccessful, as in many other bacterial genera (Smith, 1954), including also several *Streptomyces* species (Heim *et al.* 1957). There may exist in *S. olivaceus* a cytochrome substrate active for the terminal oxidase and different from the mammalian cytochrome *c* or a flavoprotein carrier directly mediating with oxygen.

SUMMARY

1. Some evidence has been presented for the occurrence of the tricarboxylic acid cycle in *Streptomyces olivaceus*. The intermediates of this cycle stimulate oxygen uptake over the endogenous respiration by the whole resting cells.

2. The cell-free extracts contained the following enzymes: aconitase, *iso*citric dehydrogenase, succinic dehydrogenase, malic dehydrogenase, glutamic dehydrogenase, alanine dehydrogenase, reduced diphosphopyridine nucleotide oxidase, reduced triphosphopyridine nucleotide–cytochrome *c* reductase and reduced diphosphopyridine nucleotide cytochrome *c* reductase; the extracts were capable of synthesizing citrate from acetate, coenzyme A, adenosine triphosphate, inorganic phosphate and oxaloacetate.

3. Activities of pyruvic and α-oxoglutaric dehydrogenases, fumarase and cytochrome *c* oxidase could not be demonstrated in the cell-free extracts.

4. Cells grown on acetate possess *iso*citratase and malate synthetase.

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An Amino Acid Sequence in the Active Centre of Phosphoglucomutase

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The presence of phosphate in phosphoglucomutase was first demonstrated by Jagannathan & Luck (1949), who also showed that it may readily be exchanged with phosphate in the substrates and that it is thus probably involved in the active centre of the enzyme. Anderson & Jollès (1957) observed that it was not released from the protein even under strong acid conditions and were able to isolate serine phosphate from the partially hydrolysed enzyme. This offered the possibility of studying the amino acid sequence around the point of attachment of the phosphate by isotopic techniques. In a preliminary study of the peptides produced from a partial hydrolysate of phosphoglucomutase labelled with ^{32}P, Koshland & Erwin (1957) and Koshland, Ray & Erwin (1958) suggested that the sequence around the serine phosphate (SerP) residue was Asp.SerP.Gly.Glu.Ala.Val. [For definitions of the abbreviations of amino acids used in this paper see *Biochem. J.* (1957), **66**, 6.] This was very similar to that found in trypsin and chymotrypsin around the serine residue that can react with di*iso*propyl phosphorofluoridate. The sequence Asp.Ser.Gly thus appeared to be a common feature of enzymes with rather dissimilar functions, and these conclusions have been discussed by several authors in connexion with the problem of the relationship between structure and function (Lumry, 1959; Koshland, 1959; Linderstrøm-Lang & Schellman, 1959; Dixon, Neurath & Pechère, 1958). In the work of Koshland & Erwin evidence for the purity of the peptides studied was lacking, and, by comparing partial hydrolysates of [^{32}P]phosphoglucomutase with similar hydrolysates of labelled chymotrypsin, we were able to show (Milstein & Sanger, 1960) that the sequence around the serine phosphate residue in phosphoglucomutase was not Asp.SerP.Gly. The present paper describes the determination of a pentapeptide sequence around the serine phosphate residue.

In most experiments with peptides of serine phosphate it is usually possible to separate them from other peptides by virtue of their strong acid group by ion-exchange methods (Flavin, 1954) or by ionophoresis at an acid pH. Since most of the labelled peptides in a partial hydrolysate of [^{32}P]-phosphoglucomutase contain a basic residue, they are neutral and purification is much more difficult. We have thus determined as much as possible of the sequence by isotopic methods, since it is relatively easy to separate the labelled peptides from one another but very difficult to separate them from the very complex mixture of non-labelled peptides that is derived from such a large protein. Some such methods have been described previously (Naughton, Sanger, Hartley & Shaw, 1960; Sanger & Shaw, 1960; Milstein & Sanger, 1960). The interrelationships of the various peptides (Tables 3 and 4) were first determined by studying the effect of partial acid hydrolysis and Edman degradation on them. The presence of a histidine residue after the serine phosphate and of an acidic residue next to the histidine was concluded from the ionophoretic rates of the peptides at different pH values.

Another method for the identification of histidine peptides was also developed by making use of the lability of the imidazole ring to photo-oxidation. Weil, Gordon & Buchert (1951) showed that some amino acids can be oxidized when they are exposed under a visible light in the presence of methylene

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blue and that peptide bonds do not participate in the photochemical action of the dye. Of the amino acids studied, the most reactive appeared to be histidine, tyrosine, tryptophan, methionine and cysteine. It was suggested that, during the reaction, the imidazole ring breaks, with formation of an aldehyde group. It would therefore be expected that the electrophoretic mobility of a peptide containing histidine will change after exposure to photo-oxidation. Since no other basic amino acid should react under those conditions, differentiation of histidine peptides from other basic amino acids containing peptides is possible.

Another specific test that proved useful was the use of periodate (Sanger & Shaw, 1960). If the ionophoretic rate of a peptide was altered after treatment with periodate it could be concluded that it contained serine or threonine in the *N*-terminal position.

Further information about the individual residues present in the peptides could be obtained by studying their R_F values on paper chromatography. Pardee (1951) showed that the chromatographic rate of a peptide in a given solvent system can be related to the chromatographic rate of the amino acid components by the following formula:

$$RT \ln (1/R_{F(p)} - 1) = (n-1)A + B + \Sigma RT \ln (1/R_{F(a)} - 1) \quad (1)$$

in which R is the molar gas constant, T the absolute temperature, n the number of amino acid residues in the peptide, and A and B are constants. $R_{F(p)}$ and $R_{F(a)}$ are the R_F values of the peptide and the amino acids respectively. This relationship has been shown to fit well with the experimental data obtained on different solvent systems with 46 peptides (Moore & Baker, 1958). These authors reported no significant differences for groups of peptides in which the sequence of amino acids was altered.

From this it follows that if, for a given solvent system, the values of the constants and the R_F values of a peptide with one unknown amino acid are known, the R_F of the unknown amino acid could be easily deduced. The R_F values of an unknown amino acid will not in most cases be enough to characterize it. However, from the combination of R_F data on more than one system and information obtained by mobility studies and specific reactions, it should be possible to obtain valuable information leading to the resolution of the sequence around one known amino acid. With these techniques it could be concluded that the most probable sequence was Thr.Ala.SerP.His.-(Glu or Asp). In order to confirm this and to identify the acidic amino acid it was necessary to purify certain of the peptides and identify the amino acids present by established techniques. A rather extensive purification was found to be necessary, but since the size and approximate composition of the peptides was known it was easy to ascertain when they were pure. From these results it was shown that the sequence was Thr.Ala.SerP.His.Asp.

METHODS

Phosphoglucomutase (PGM) was prepared by the method of Najjar (1948) and dialysed against distilled water before use. Trypsin and chymotrypsin were crystalline salt-free preparations from Worthington Biochemical Corp. Elastase was a gift from M. A. Naughton (Naughton & Sanger, 1960).

Preparation of [^{32}P]*phosphoglucomutase*

Two methods were used for the preparation of [^{32}P]PGM. In method 1 PGM was labelled by allowing it to react with glucose 6-[^{32}P]phosphate, which was prepared through the hexokinase reaction. A mitochondrial suspension was used to generate adenosine [^{32}P]triphosphate from [^{32}P]phosphate. In method 2 glucose 1-[^{32}P]phosphate was prepared by the action of potato phosphorylase on starch in the presence of [^{32}P]phosphate, and allowed to react with the PGM.

Method 1. Each 8·2 ml. of the incubation mixture contained (amounts in μmoles unless otherwise stated): $MgSO_4$, 60; glucose, 225; 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, 125; glutamate or proline, 75; KCl, 250; adenosine triphosphate (ATP), 2; $H_3{}^{32}PO_4$ (carrier-free), 0·2–2 mc; fresh mitochondria suspension, 0·75 ml.; and excess of hexokinase. The mitochondria were freshly prepared from sucrose homogenates of rat liver and tested before use. Yeast hexokinase was prepared according to Berger, Slein, Colowick & Cori (1946), by using the preparation of stage 5. The total volume of the incubation mixture was 8·2 ml. and the pH 7·3. After the addition of the hexokinase, the mixture was kept for 40 min. at room temperature. Histidine (75μmoles, pH 7·5) was then added together with a dialysis bag containing a solution of PGM in a small volume (0·5–5 ml.); the whole was allowed to equilibrate in the cold overnight. The dialysis bag was then transferred to a 5 l. beaker and dialysed against distilled water with stirring. After 12 hr. the water was changed, and the dialysis repeated five times, unless otherwise indicated. Even after additional changes of the water, considerable amounts of radioactive phosphate and glucose phosphate were released when the protein was subjected to tryptic digestion or partial acid hydrolysis and to ionophoresis at pH 6·5 or 3·5. This preparation was used for the structural studies, unless otherwise indicated. The specific activity of the protein ranged from about 0·05 to 0·5μc/mg.

As a control in one experiment this procedure was repeated exactly as above, except that PGM was denatured before use. The denatured PGM was prepared by precipitating 2 mg. of protein with 10% trichloroacetic acid and then dialysing it against 1·5 l. of distilled water. Native enzyme (5 mg.) was included in the same experiment. After the usual procedure (except that a smaller amount of ^{32}P was used) the native enzyme contained 7·5μmc/mg. of PGM, whereas the denatured one contained 0·3μmc/mg. of protein. As will be seen below, in the denatured preparation, only phosphate and glucose phosphate were detected

after partial acid hydrolysis or tryptic digestion, whereas the native preparation contained phosphopeptides in addition.

Method 2. A crude phosphorylase preparation was prepared by blending 20 g. of potatoes with 20 ml. of water. Kaolin (0·6 g.) was added and the mixture centrifuged. To 5 ml. of 2·5% starch solution were added 0·5 ml. of 0·2M-phosphate buffer, pH 7·0, 1·5 ml. of the above supernatant solution, a few drops of toluene and 1 mc of $H_3{}^{32}PO_4$ (carrier free). The mixture was kept at room temperature for 8 hr. A solution of 5 mg. of PGM in 1·0 ml. of water containing histidine (0·04M, pH 7·5) and $MgSO_4$ (2·5 mM) was dialysed overnight against the above mixture. The dialysis bag was then placed in a 5 l. beaker and dialysed against several changes of water.

Tryptic hydrolysis of [^{32}P]phosphoglucomutase

[^{32}P]PGM was twice dialysed against 40% urea and then against water until the urea was removed. Under these conditions the denatured PGM was precipitated. The precipitate was suspended in $(NH_4)_2CO_3$ (0·5%) to produce a 0·25% suspension, and crystalline trypsin (7·5μg./ml.) was added. Incubation was carried out at 37° with occasional stirring. Samples were removed at various times and subjected to ionophoresis after removal of the $(NH_4)_2CO_3$ *in vacuo*.

Partial acid hydrolysis. Two methods of partial acid hydrolysis were used:

(*a*) 5·7N-HCl at 100°. In general the reaction was carried out in small test tubes with approx. 0·2 ml. of acid. The time of hydrolysis was 30 min. unless otherwise indicated.

(*b*) 12 N-HCl at 37°. This method was used principally when larger amounts of protein were hydrolysed. Between 20 and 70 mg. of protein was incubated with 3–10 ml. of 12 N-HCl, for 30–40 hr. at 37° in tubes fitted with ground-glass stoppers.

Ionophoretic fractionation of peptides

The peptides were fractionated by high-voltage paper ionophoresis as previously described (Michl, 1951; Naughton *et al.* 1960). The buffer at pH 4·0 was prepared by adding pyridine to the buffer at pH 3·5. Unless otherwise stated, Whatman no. 52 paper was used with voltage gradients of 40 v/cm.

The pH–mobility curves (Fig. 9) were determined in the apparatus of Gross (1955) with Whatman no. 1 filter paper and 50 v/cm. (Naughton *et al.* 1960).

In order to standardize the positions of peptide spots and the conditions of each ionophoretic run, it was found convenient to use a mixture of coloured markers, which were usually applied at one side of the paper and run in parallel with the radioactive peptides. A suitable mixture for running at pH 3·5 or 6·5 towards the anode was made by using the following dyes: Pappenheim's panoptic stain; isamine blue; xylene cyanol FF; phenol red; methyl orange; safranine; Bordeaux; methyl green; light green SF.

For two-dimensional ionophoresis the following method, which was preferable to those previously described (Naughton *et al.* 1960), was used. Ionophoresis was carried normally in the first direction and the paper was radioautographed. A strip containing the peptides was cut out and sewn with a sewing machine to a second strip of paper. The paper was then wetted with the buffer to be used in the second dimension in such a way that it flowed evenly towards the strip from each side, thus sharpening the bands. The paper was then subjected to ionophoresis in the second dimension.

Paper chromatography

Two systems were used for studying the R_F values of the peptides: butan-1-ol–acetic acid–water–pyridine (30:6:24:20, by vol.) (Waley & Watson, 1953); phenol–water: phenol saturated with water at 25°. Whatman no. 52 filter paper was used with both systems.

Photo-oxidation technique for the detection of histidine peptides

The method was tested first with pure amino acids. The following amino acids were applied on a sheet of Whatman no. 52 paper, in two series one next to the other: tryptophan, tyrosine, arginine, lysine, histidine and methionine. The paper was moistened with pyridine–acetate buffer (10% of pyridine, 0·4% of acetic acid, pH 6·5) and then with methylene blue (0·2% in absolute ethanol). The paper was then covered with a polythene sheet, and one of the series of amino acids covered with a black paper to use as control. The paper was pressed between glass plates and the part containing both series of amino acids put under a 150 w lamp at 30 cm. for 2 hr. It was then dried in the dark, the amino acids were subjected to ionophoresis at pH 2·1 for 20 min. and the paper was sprayed with ninhydrin. The methylene blue, which covers part of the sheet, does not seem to interfere with the ninhydrin reaction and the spots can easily be located. It was observed (Fig. 1) that tryptophan, present in the control, completely disappeared after photo-oxidation. Histidine, which ran together with the basic amino acids in the control, is slightly slower than tyrosine after the treatment. No difference was detected in the other amino acids tested.

The test for histidine peptides was made as follows: a partial acid hydrolysate of [^{32}P]PGM containing about 2·5μmc of ^{32}P was applied on Whatman no. 52 paper in a 1·5 cm. wide band and subjected to ionophoresis at pH 4·0

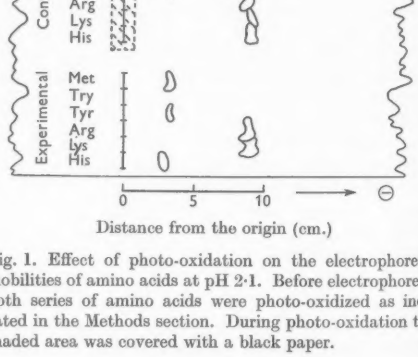

Fig. 1. Effect of photo-oxidation on the electrophoretic mobilities of amino acids at pH 2·1. Before electrophoresis both series of amino acids were photo-oxidized as indicated in the Methods section. During photo-oxidation the shaded area was covered with a black paper.

for 2 hr. It was then radioautographed, and a strip was cut to include peptides 7, 6, 5 and 4, and part of the glucose [^{32}P]phosphate to be used as a marker. The strip was moistened with buffer solution and methylene blue, and placed under the 150 w lamp in a dark room. Another strip of the same paper containing histidine, lysine and arginine, covered and uncovered with black paper, was subjected to the same treatment and used as further control. Both strips were placed together under the same light, and exposed for 80 min. After being dried in the dark, they were sewn with a sewing machine on a sheet of Whatman no. 52 paper at the same distance from the edge of the paper as the hydrolysate was applied in the first run. Ionophoresis was repeated under the same conditions (pH 4·0, 2 hr.).

Direct identification of amino acids in peptides

In order to obtain sufficient quantities of the peptides to identify the amino acids present in them directly, larger-scale experiments were carried out: [^{32}P]PGM (70 mg.) was hydrolysed for 2 days at 37° in 12N-HCl and passed through a column (20 cm. × 0·8 cm. diam.) of Dowex 50X4 (200–400 mesh). About 50% of the radioactivity passed straight through the column when washed with water and appeared in the first 20 ml. of eluate. This was collected, freeze-dried and labelled F_1. The column was then developed with ammonium acetate solution (2%, pH 3·7), which eluted about 30% of the radioactivity. This fraction, which contained most of the material in the partial hydrolysate, was freeze-dried and labelled F_2. No more radioactivity could be eluted from the column by increasing the pH to 7·0.

Samples of fractions F_1 and F_2 were subjected to ionophoresis at pH 4·0 and 6·5. The bands obtained were designated by numbers as shown in Fig. 4. F_1 contained the acidic components, namely bands 2 (serine phosphate), 4 and 5 as well as 1 (phosphate) and 3 (glucose phosphate). F_2 contained the 'neutral' components (bands 6 and 7). This fractionation on Dowex 50 was very useful for the purification of peptide 5 but unnecessary for the 'neutral' peptides.

All the peptides were purified further by paper ionophoresis and chromatography (Table 6), generally with the two-dimensional method. Whatman no. 52 paper was used where possible, but, when the amount of material was so large that it would have caused overloading on no. 52, it was applied to no. 3 MM paper.

The first run of fraction F_2, or when the Dowex 50 step was omitted, was made on Whatman no. 3 MM paper (peptides 6 and 7, Table 6) and the bands were eluted and dried. In other cases the samples were applied as 2 cm. bands and run in two dimensions. After location of the spots by radioautography they were cut out and were either eluted and hydrolysed for amino acid determination or sewn on another sheet of paper and again run in two directions.

The amino acids were identified by two-dimensional paper electrophoresis (pH 2·1; 30 min.; 80 v/cm.) and chromatography in butanol–acetic acid–water (3:1:1, by vol.) for 3 hr. (L. F. Smith, unpublished work), except for 7A and 7C (Table 6), in which case only electrophoresis was used.

RESULTS

Labelling of phosphoglucomutase

According to Kennedy & Koshland (1957) PGM is inhibited by di*iso*propyl phosphorofluoridate (DFP). An attempt was therefore made to label the enzyme by treatment with [^{32}P]DFP. The conditions used were similar to those used by the above authors for obtaining 100% inhibition of PGM in 15 min.: PGM was incubated with [^{32}P]-DFP (1·0 mM), cysteine (25 mM), $MgSO_4$ (1·5 mM) and glucose 1-phosphate (5 mM). The pH was adjusted to 8·2. After 3 hr. the mixture was dialysed extensively against water. A partial acid hydrolysate of the freeze-dried protein was examined by ionophoresis at pH 3·5 followed by radioautography. No 'phosphopeptides' or 'phospho-amino acids' were detected but only inorganic phosphate and a trace of mono*iso*propyl phosphate. A second experiment in which the concentration of the DFP was increased ten times, the incubation period increased to 12 hr. and the enzyme precipitated with HCl before dialysis to avoid the possibility of removal of the phosphate during dialysis, was also negative.

Labelling the PGM by exchange of phosphate with labelled substrate proved much more successful. According to Kennedy & Koshland (1957) their preparation contained about 1·5 μmc/mg. of protein, by using as starting material 1 mc of ^{32}P. This would have been insufficient for our purposes. With the techniques described above, it was possible to prepare a labelled protein containing as much as 0·5 μc/mg. This offered the possibility of using minute amounts of protein for sequence studies.

Stability of the bound ^{32}P in labelled phosphoglucomutase. The [^{32}P]PGM prepared by these methods still contained some diffusible ^{32}P. When dialysed five times against 100 ml. of water for 24 hr., some radioactivity still diffused out. The material after such treatment was dialysed under various conditions and the amount of radioactivity that diffused out was measured (Table 1). Three separate experi-

Table 1. *Removal of ^{32}P from [^{32}P]phosphoglucomutase by dialysis*

Expt.	Dialysis against	Amount diffused out (%)
A	Water	5·6
	Water	6·1
	Water	6·0
B	0·1 mM-Phosphate (pH 6·0)	1·5
	4·0 mM-Phosphate (pH 6·2)	3·7
	Water	1·0
C	40% Urea	7·1
	Water	1·3
	Water	1·2

ments were performed, in which samples containing about 50 μmc of [^{32}P]PGM were dialysed against 25 ml. of the indicated solution for 24 hr. The radioactivity of the dialysate was determined and the dialysis was repeated with a further 25 ml. of solution. In all cases some radioactivity diffused out, although in a small proportion compared with the total amount. When the urea-treated enzyme (Table 1, Expt. C) was digested with trypsin and the hydrolysate subjected to ionophoresis at pH 6·5, a spot corresponding to phosphate and amounting to about 30 % of the total remaining radioactivity was obtained. This suggests that there is still some labile phosphate remaining inside the sac even after very extensive dialyses, namely three times against 5000 vol. of water (12 hr. each with stirring), five times against 100 vol. of water (24 hr. each), once against 16 vol. of 40 % urea (24 hr.) and finally twice against 16 vol. of water for 24 hr.

Tryptic hydrolysate of [^{32}P]*phosphoglucomutase*

Fig. 2 indicates the time course for the hydrolysis of [^{32}P]PGM with trypsin. The results clearly suggest that band B (not necessarily a single radioactive peptide) is the primary breakdown product formed by the splitting of very susceptible bonds, the reaction being complete when all the denatured material has come into solution (usually less than 4 hr.). A second slower reaction takes place and a radioactive peptide is released from band B which moves quicker towards the anode at pH 6·5 (band A). This second reaction hydrolyses band B after incubation for 17 hr. A third product, band C, begins to be apparent at that time. Trypsin usually contains traces of other pancreatic enzymes, especially chymotrypsin, and it is possible that band C is due to the action of such a contaminant on band A or B. Partial acid

Fig. 2. Tracing of radioautograph obtained after ionophoresis (pH 6·5; 40 v/cm.; 2 hr.) of tryptic digest of [^{32}P]PGM. Conditions of the digestion were as described in text. Times of digestion: *a*, 1½ hr.; *b*, 4 hr.; *c*, 7 hr.; *d*, 17 hr.; *e*, markers of bands 1 and 3 (Fig. 4).

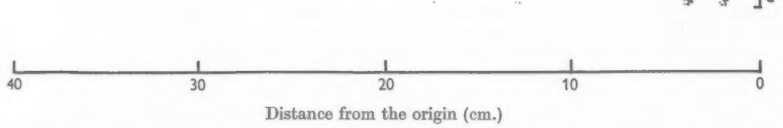

Fig. 3. Tracing of radioautograph obtained after ionophoresis (pH 3·5; 40 v/cm.; 2 hr.) of partial acid hydrolysates of tryptic peptides (Fig. 2) derived from [^{32}P]PGM prepared by methods 1 and 2. *a*, Band B (method 1); *b*, band A (method 1); *c*, band A (method 2); *d*, band B (method 2).

hydrolysates of the three bands were made and since no difference of pattern was observed, either the pure bands or a mixture of them was used in further partial hydrolysis studies.

The [^{32}P]PGM prepared by method 2 was also subjected to hydrolysis by trypsin. Fig. 3 shows an ionogram of the partial acid hydrolysate of the tryptic bands, which was run together with a similar hydrolysate of [^{32}P]PGM prepared by method 1. The exact matching of the bands indicates that both methods of labelling are equivalent.

As a control a sample of PGM that had been denatured before the labelling procedure was also subjected to tryptic digestion. The small amount of radioactivity which remained associated with the protein after dialysis gave after digestion a spot moving with phosphate and a trace of a second spot moving with glucose phosphate on ionophoresis at pH 6·5.

Partial acid hydrolysis of [^{32}P]*phosphoglucomutase*

Fig. 4 shows the pattern obtained when [^{32}P]-PGM was subjected to partial acid hydrolysis (5·7 N-HCl; 100°; 30 min.) and ionophoresis at pH 3·5. Band A from the tryptic digest (Fig. 2) was similarly hydrolysed and run side by side. As a control, denatured PGM was also subjected to the same treatment. For comparison the pattern obtained from di*iso*propoxy[^{32}P]phosphinylchymotrypsin, which had been subjected to the same treatment, is shown. It is quite clear that the main features in the patterns of PGM and chymotrypsin are different, so that it may be concluded that PGM does not contain the sequence Gly.Asp.SerP.Gly which is in chymotrypsin.

Band 1 runs in the position of inorganic orthophosphate and band 2 was identified as serine phosphate by comparison with a known marker on ionophoresis at pH 3·5 and 2·1.

Band 3 appeared to be an impurity, which can be released during the isolation of the tryptic peptide. A sample eluted from band 3 was run in parallel with glucose 6-phosphate in ionophoresis at pH 3·5. The paper was treated with phosphate reagent (Burrows, Grylls & Harrison, 1952) and then radioautographed. There was a satisfactory match between the blue colour and the radioactive spot. It thus seems likely that it is a glucose phosphate, and the known stability of the phosphate residues would suggest that it is glucose 6-phosphate rather than glucose 1-phosphate.

Bands 6 and 7 are mixtures of a number of components which can be separated at pH 2·1 or 6·5. The ionophoresis of the peptide mixture at pH 6·5 is shown in Fig. 5. Fig. 6 shows a two-dimensional ionogram run at pH 3·5 and 6·5, and shows the relationships between the patterns obtained at the two pH values. At pH 6·5 bands 4 and 5 are contaminated by a 'tailing' of band 6A and can best be purified at pH 3·5, whereas the other peptides are present as mixtures (bands 6 and 7) at pH 3·5 and are best purified at pH 6·5. The proportions of the peptides in hydrolysates obtained by two different methods were determined by measuring the relative degree of darkness of the radioautographs of the ionograms run at pH 6·5. The measurements were carried out in a Chromatograph automatic recording reflective densitometer (Joyce, Loebel and Co. Ltd., A8, Princeway Team Valley, Gateshead, Co. Durham) and the results are shown in Table 2.

The time course of the hydrolysis was followed by ionophoresis at pH 3·5 and 6·5. A sample of the tryptic bands was hydrolysed with 5·7 N-HCl in a boiling-water bath and samples were taken out at different times and subjected to ionophoresis at pH 3·5. The relative amounts of each peptide were determined by densitometry of the radioautographs. Fig. 7 shows clearly that bands 7, 2 and 1 increased continuously. Bands 5 and 6, on

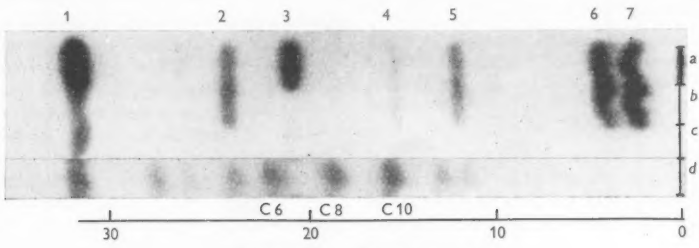

Fig. 4. Radioautograph obtained after ionophoresis (pH 3·5; 40 v/cm.; 2 hr.) of partial acid hydrolysates of ^{32}P derivatives. *a*, [^{32}P]PGM; *b*, band A (Fig. 2); *c*, denatured PGM subjected to the same labelling and subsequent treatment as *a*; *d*, di*iso*propoxy[^{32}P]phosphinylchymotrypsin; the Asp.SerP (C6), Asp.SerP.Gly (C8), and SerP.Gly (C10) bands are indicated (Naughton, Sanger, Hartley & Shaw, 1960).

the other hand, pass through a maximum. Band 4 does not seem to change much after reaching a low level. The time course of the hydrolysis was als followed at pH 6·5. In this case two different hydrolysis conditions were used: 5·7 N-HCl and 12 N-HCl. The time course of the hydrolysis is more or less similar for both conditions, when 10 min. hydrolysis in the 5·7 N-HCl is compared with 24 hr. in 12 N-HCl as suggested by Naughton *et al.* (1960). Bands 6A and 7A are the main ones present after 30 min. in 5·7 N-HCl (or 72 hr. in 12 N). Band 7A is, however, still increasing whereas band 6A is beginning to diminish. Both seem to be quite stable peptides, especially the former.

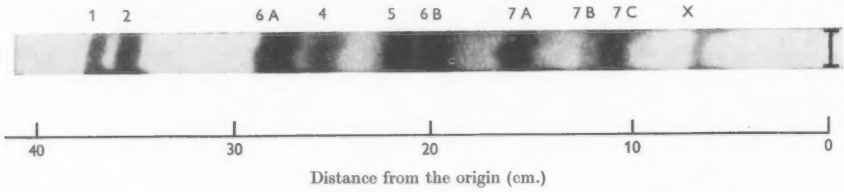

Fig. 5. Radioautograph obtained after ionophoresis (pH 6·5; 40 v/cm.; 2 hr.) of the partial acid hydrolysate of band B (Fig. 2).

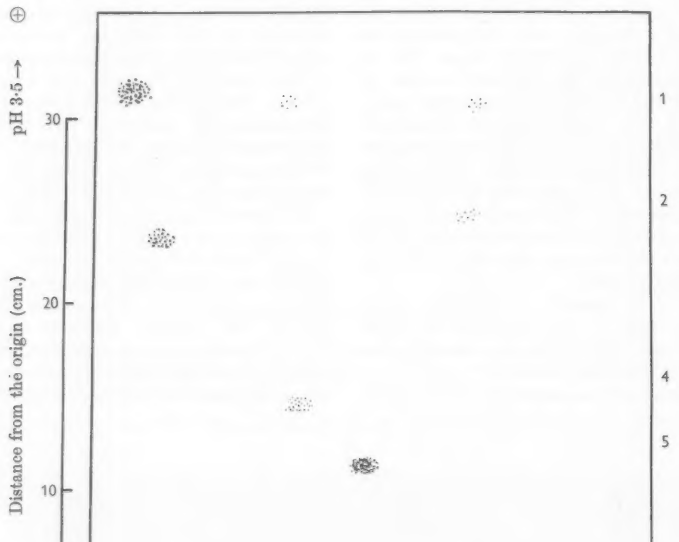

Fig. 6. Tracing of radioautograph of a two-dimensional ionogram (pH 3·5 and 6·5; 40 v/cm.; 2 hr. and 110 min. respectively) of the partial acid hydrolysate of a mixture of isolated bands from the tryptic digest of [^{32}P]PGM.

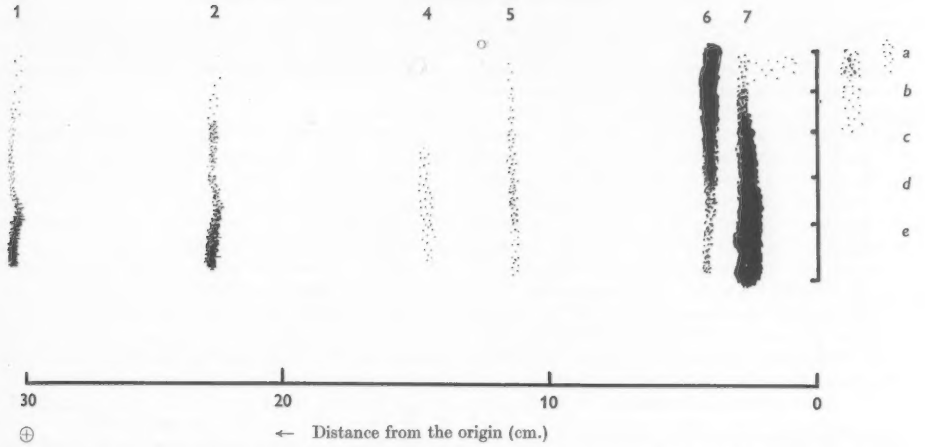

Fig. 7 (*a*). Tracing of radioautograph obtained after ionophoresis (pH 3·5; 40 v/cm.; 2 hr.) of the partial hydrolysis of band A (Fig. 2) for different times: *a*, 5 min.; *b*, 10 min.; *c*, 20 min.; *d*, 30 min.; *e*, 45 min.

Table 2. *Yields of peptides* (%) *from partial acid hydrolysis of* [^{32}P]*phosphoglucomutase*

Band no.	5·7 N-HCl for 30 min. at 100°	12 N-HCl for 3 days at 37°
1	14·0	5·5
2	15·6	11·6
4*	2·5	2·6
5	8·2	10·8
6A	18·4	26·8
6B	3·9	4·6
7A	24·4	28·6
7B	1·7	1·2
7C	6·2	7·7
Others	4·7	1·2

* Contaminated with material running as band 6 at pH 3·5.

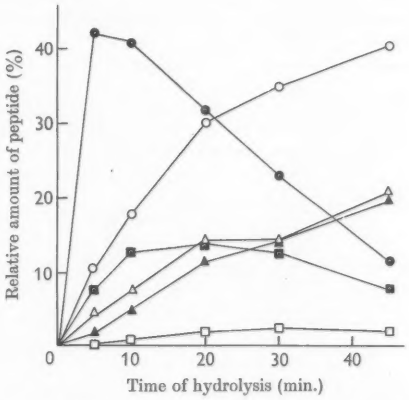

Fig. 7 (*b*). Relative amounts of each band during the hydrolysis, calculated by densitometry of the radioautograph [Fig. 7 (*a*)]. △, Band 1; ▲, band 2; □, band 4; ■, band 5; ●, band 6; ○, band 7.

Structure of the peptides

In order to determine the interrelations of the various bands they were subjected to a further partial acid hydrolysis (5·7 N-HCl; 100°; 45 min.) and the products identified by ionophoresis together with markers of the original partial acid hydrolysate. The results are shown in Table 3. Table 4 shows the products produced when the various bands were subjected to the Edman degradation (Naughton *et al.* 1960).

Peptide 7A gives only serine phosphate on partial hydrolysis, suggesting that it is a dipeptide, and, since phosphate and not serine phosphate was obtained as the main product after the Edman degradation, the serine phosphate is *N*-terminal. Peptide 6A also has *N*-terminal serine phosphate and gives 7A on partial hydrolysis, indicating that it is the tripeptide formed by addition of a residue to the *C*-terminal end of 7A. Peptide 5 gives 4 and serine phosphate on partial hydrolysis. It does not give 7A and therefore the serine phosphate must be the *C*-terminal residue. It is converted into 4 by the Edman degradation. It is thus most probably a tripeptide and peptide 4 the other dipeptide. The yield of band 4 is very low and it was not possible to carry out the re-hydrolysis and Edman degradations with it. The various relationships of the

Table 3. *Products of the rehydrolysis of radioactive bands*

Bands 1, 2, 4 and 5 were isolated by ionophoresis at pH 3·5, the other bands at pH 6·5, and hydrolysed in 5·7N-HCl for 45 min. at 100°. They were again subjected to ionophoresis at the same pH used for their isolation and identified by their position with respect to markers of the original partial hydrolysate. The relative amount of each product is indicated with crosses; tr., trace.

Band rehydrolysed (Figs. 4 and 5) ...	1	2	4	5	6A	7A	7B	7C	X
1	×	.	.	.	.	.	.	.	.
2	×	× ×	.	.	.	.	.	.	.
4	×	× ×	× × ×	.	.	.	.	.	.
5	× ×	× × ×	×	×	.	.	.	.	.
6A	×	× ×	.	.	× ×	× × × ×	.	.	.
6B	×	× ×	×	× ×	× ×	× × × ×	tr.	tr.	.
7A	×	×	.	.	.	× × × ×	.	.	.
7B	×	× ×	× × ×	.	.	× × × ×	× × ×	.	.
7C	×	× × ×	×	×	.	× × × ×	×	×	.
X	×	×	.	.	.	× × × ×	.	.	× ×

Table 4. *Products of the Edman degradation of radioactive bands*

Band 5 was isolated at pH 3·5 and bands 6A and 7A at pH 6·5 as indicated in Figs. 4 and 5 respectively. The bands were eluted and subjected to the Edman degradation followed by ionophoresis at the same pH used for their isolation. Identification of the products was made by their position with respect to markers of the original partial hydrolysate. The relative amount of the products is indicated with crosses; tr., trace.

Band	Products
5	1 (×)+4 (× × ×)+5 (× ×)
7A	1 (× × × ×)+2 (tr.)+a* (× ×)+7A (× ×)
6A	1 (× × ×)+2 (tr.)+6A (× × × ×)

* Unknown spot, running between 5 and 7A.

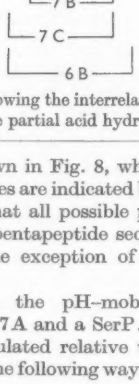

Fig. 8. Diagram showing the interrelationships of peptides of the partial acid hydrolysate.

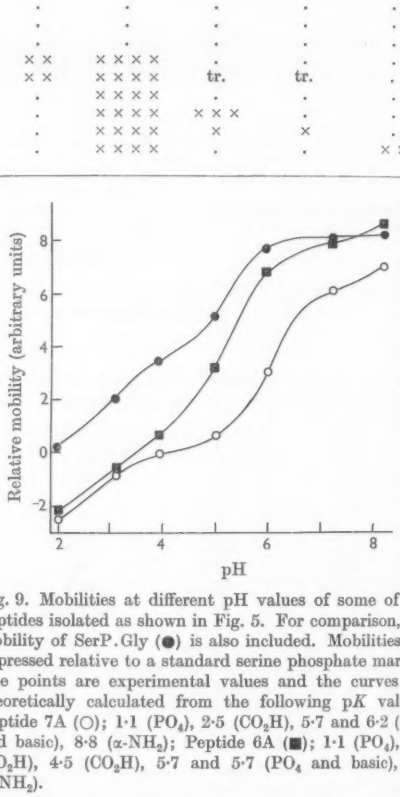

Fig. 9. Mobilities at different pH values of some of the peptides isolated as shown in Fig. 5. For comparison, the mobility of SerP.Gly (●) is also included. Mobilities are expressed relative to a standard serine phosphate marker. The points are experimental values and the curves are theoretically calculated from the following pK values: Peptide 7A (○); 1·1 (PO_4), 2·5 (CO_2H), 5·7 and 6·2 (PO_4 and basic), 8·8 (α-NH_2); Peptide 6A (■); 1·1 (PO_4), 2·5 (CO_2H), 4·5 (CO_2H), 5·7 and 5·7 (PO_4 and basic), 8·8 (α-NH_2).

peptides are shown in Fig. 8, where the unknown amino acid residues are indicated by A, B, X and Y. It can be seen that all possible partial hydrolysis products of the pentapeptide sequence have been detected with the exception of the tetrapeptide B.SerP.X.Y.

Fig. 9 shows the pH–mobility curves for peptides 6A and 7A and a SerP.Gly marker. The results were calculated relative to a serine phosphate marker in the following way: a titration curve of serine phosphate was drawn, and, assuming mobility to be proportional to net charge, an arbitrary mobility curve was obtained by substituting the alkali volume by an arbitrary centimetre scale. Glucose markers were used to correct for the displacement of the origin due to endosmosis. The relative mobility for a peptide at a given pH is equal to: (Distance of peptide from glucose marker)/(distance of SerP from glucose marker) × arbitrary mobility of SerP at same pH (Naughton *et al.* 1960). Values for band 3 (glucose phosphate) were also determined and showed good agreement with the theoretical curve calculated

from the known pK values. At pH 4·0, SerP.Gly has one negative charge, whereas the dipeptide 7A is neutral. This suggests that the unknown amino acid called X in Fig. 8 is a basic one. Comparing the increase in rates of SerP.Gly and peptide 7A between pH 4·0 and pH 7·2, it can be seen that although the molecular weight of peptide 7A is greater, its mobility has increased more than the increase in mobility of SerP.Gly, suggesting another group ionizing near the range of the second phosphate. Fig. 9 also shows the theoretical curve, based on ionizable groups in peptide 7A, including a basic group with a pK of 5·7 or 6·2. It can be seen that this curve fits the experimental data. The tripeptide 6A is more acidic than 7A at pH values higher than 3·5. At pH 4·5, when the second phosphate group is completely discharged, the tripeptide is clearly acidic. The inclusion of an acidic group with pK 4·5, and slight changes in the pK of the phosphate or basic, or both, groups of peptide 7A, fit in with the experimental data. The mobility data, strongly suggest then, the presence of a basic amino acid (pK between 5 and 7) and an acidic amino acid (pK about 4·5) on the C-terminal side of serine phosphate. Peptides 4 and 5 run at both pH values (3·5 and 6·5), as would be expected for a di- and tri-peptide having neutral amino acids and a C-terminal serine phosphate.

The above curves indicate that residue X (Fig. 8) is a basic amino acid having a pK about 6·5 and therefore histidine. This was further confirmed by photo-oxidation (see below) and by the following experiment: band 7 (3 μmc) isolated by ionophoresis at pH 3·5 was added to 3 mg. of a hydrolysate (5·7 N-HCl; 100°; 1 hr.) of unlabelled PGM and subjected to ionophoresis at pH 3·5 (2 hr.; Whatman no. 1 paper). The radioactive band was cut out, eluted and re-run at pH 6·5 for 90 min. and then at pH 2·1 for 50 min. The paper was then radioautographed and sprayed with Pauly reagent (Fraenkel-Conrat & Singer, 1956). A faint but distinct red spot fell in exactly the same position as the radioactivity. Since the possibility of a histidine peptide contamination having the same mobility as peptide 7A in the three different systems used seems unlikely, the assumption that SerP.His is one of the dipeptides of the partial hydrolysis of PGM seems reasonable.

Fig. 10 shows the results of the photo-oxidation experiment. The pattern obtained in the first run is shown at the origin. After photo-oxidation and re-running in the second dimension but with the same buffer, glucose phosphate and peptides 4 and 5 are mostly unchanged, whereas most of peptides 6 and 7 are in a new position, indicating an increase in negative charge. Some of the unchanged peptides are still present. Band 6 shows two unchanged spots, and two corresponding faster-moving spots (presumably 6A, 6B and their oxidized derivatives) and some free phosphate. Band 7 gave one spot in the position of the unchanged material and several other spots. The strongest one, running in a position between 4 and 5, is probably the oxidized 7A, and the slower-moving spots the other peptides present in 7 which can be separated at pH 6·5. There are also a phosphate spot and three very faint spots whose nature is unknown. It is quite clear from this experiment that the main components in 6 and 7 are histidine peptides. The presence of spots in the positions of the unchanged peptides from 6 and 7 suggests that in these conditions the reaction is not quantitative. In this respect it has to be considered that 10% of unchanged histidine would hardly be detected in the ninhydrin control experiment, but would give a very clear spot in the radioactive experiment.

In order to identify the presence of N-terminal serine or threonine residues, peptides 4 and 5 were

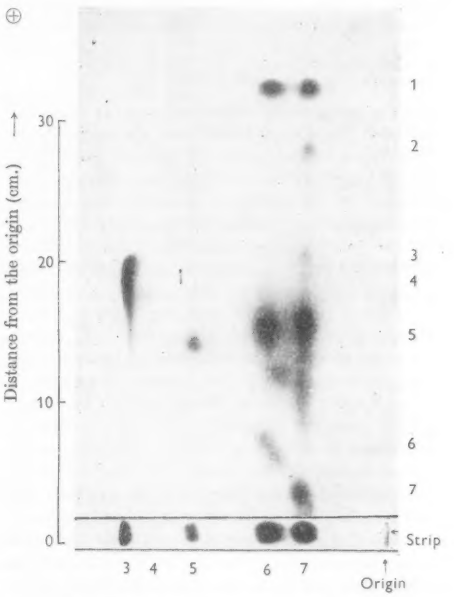

Fig. 10. Radioautograph obtained after ionophoresis of the photo-oxidized peptides. A paper strip obtained by ionophoresis (pH 3·5; 40 v/cm.; 2 hr.) was subjected to photo-oxidation and then sewn to another sheet of Whatman no. 52 filter paper and subjected to ionophoresis in the second dimension with the same conditions. The photograph also shows the radioautograph of the photo-oxidized strip, and the position in which it was sewn before ionophoresis in the second dimension.

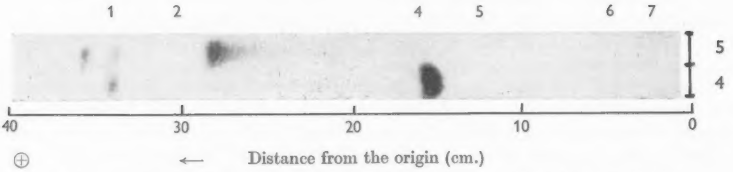

Fig. 11. Radioautograph obtained after ionophoresis (pH 3·5; 40 v/cm.; 2 hr.) of the periodate-treated peptides 4 and 5 from Fig. 3.

Table 5. *Chromatographic constants of amino acids and peptides in butanol–acetic acid–water–pyridine*

R_F values were obtained after development for 48 hr. Values of $A+B$ and of $2A+B$ were calculated from equation (1).

Sample	R_F	$A+B$
Arginine	0·13	.
Histidine	0·13	.
Glutamic acid	0·14	.
Aspartic acid	0·09	.
Proline	0·25	.
Glycine	0·12	.
Alanine	0·19	.
Valine	0·36	.
Leucine	0·55	.
Threonine	0·23	.
Serine	0·12	.
Serine phosphate	0·03	.
Leu.SerP	0·18	−1010
Gly.SerP	0·03	−1140
SerP.Gly	0·04	−1290
SerP.Ala	0·07	−1360
Ala.SerP	0·06	−1250
Glu.SerP	0·05	−1360
Thr.SerP	0·05	−1325
Mean value ($A+B$)		−1240±125
Peptide 7A (SerP.His)	0·04	−1270
		$2A+B$
Leu.Gly.Gly	0·40	−1940
Gly.Gly.Gly	0·09	−2050
Mean value ($2A+B$)		−1990
Peptide 4	0·06	.
Peptide 5	0·08	.
Peptide 6A	0·03	.

treated with periodate (Sanger & Shaw, 1960). The result is shown in Fig. 11. Peptide 5 has completely disappeared and has been replaced by a main spot moving faster than peptide 4 and two minor spots. Peptide 4, on the other hand, has remained largely unchanged. This indicates that peptide 5 has a *N*-terminal serine or threonine residue, whereas peptide 4 has not. The sequence around the serine phosphate residue may thus now be written:

$$\binom{\text{Thr}}{\text{Ser}}.\text{B.SerP.His.}\binom{\text{Asp}}{\text{Glu}}$$

where B is some neutral amino acid other than threonine or serine.

Table 5 shows the R_F values in one solvent system for a number of peptides of known constitution and for the main radioactive bands. The values of the constant $(n-1)A+B$ [equation (1)] for the known peptides have also been calculated. For the butanol–acetic acid–water–pyridine system the values for the constant are within the expected limit of errors, whereas for the phenol–water system there was much more spreading of the values. Much more reliance therefore can be placed on the values obtained in the former system.

The R_F of peptide 4 in the butanol–acetic acid–water–pyridine system was 0·06. Assuming a value of -1240 for the constant $(n-1)A+B$ it can be calculated from equation (1) that the R_F of the unknown amino acid B is 0·20. If we assume that the error in R_F is not more than 0·05 (Moore & Baker, 1958), the R_F of B must lie between 0·15 and 0·25. The only neutral amino acids to be considered are alanine, proline and possibly threonine (R_F 0·23). The results with the periodate treatment indicate that it is not threonine. Alanine and proline have very different R_F values on the phenol–water system (0·49 and 0·81 respectively), although there was considerable variation in the values of $(n-1)A+B(-300\pm260)$ in this system it seems reasonably certain that the unknown amino acid is not proline. Proline has an R_F slightly higher than leucine, whereas peptide 4 (R_F 0·06) moves much more slowly than Leu.SerP (R_F 0·37). Further evidence that peptide 4 was Ala.SerP was obtained by comparison with a sample of radioactive Ala.SerP prepared by another method from ovalbumin (D. C. Shaw, personal communication). SerP.Ala was obtained from a partial acid hydrolysate of ovalbumin (Flavin, 1954) and partly converted into Ala.SerP by inversion of the dipeptide sequence in hot dilute acid. This material ran at the same rate as peptide 4 on ionophoresis at pH 6·5 for 1 hr. 45 min. at 40 v/cm.

Assuming that peptide 4 was Ala.SerP, it was calculated that the R_F of residue A (Fig. 8) was 0·27. The periodate reaction indicated that it is either serine or threonine: serine has R_F 0·12 and threonine 0·23, suggesting that the residue is most

Table 6. *Amino acid composition of purified peptides*

Figures in parentheses represent the approximate amount present as estimated visually from the strength of the ninhydrin colour; tr., trace.

	Purification procedure					
Peptide	Chromatography on Dowex 50	Ionophoresis pH 2·1	Ionophoresis pH 3·5	Ionophoresis pH 6·5	Chromatography in butanol–acetic acid–water (3:1:1, by vol.)	Amino acid composition
5	+	.	+	.	.	Glu (3), Ser (2), Ala (2), Thr (1), Val (1), Ileu (1), Leu (1)
5	+	+	+	.	+	Ala (1), Ser (1), Thr (1)
7A	+	+	+	+	.	His (2), Ser (2), Glu (1), Asp (1), Thr (tr.), Ala (tr.)
7B	+	+	+	+	.	His (1), Ala (1), Ser (1), Thr (tr.), Glu (tr.), Asp (tr.)
6A	.	+	+	+	+	His (1), Ser (1), Asp (1), Glu (tr.), Ala (tr.), Gly (tr.)
6A	+	+	+	+	.	His (1), Ser (1), Asp (1), Gly (1), Glu (0·5)

probably threonine. From the R_F of peptide 6A in butanol–acetic acid–water–pyridine a value of 0·18 for the acidic residue (Y in Fig. 8) was calculated. Glutamic acid has R_F 0·14 and aspartic acid 0·09. This result would suggest that glutamic acid is the most probable. However, further work (see below) showed that residue Y was in fact aspartic acid. It seems probable that equation (1) will be less reliable where amino acids with charged side chains are concerned, since variations in pK values between amino acids and peptides would be expected to affect the partition coefficients, whereas this is not taken account of in the equation.

It may be concluded from the above results that the sequence around the serine phosphate residue is most probably:

$$\text{Thr.Ala.SerP.His.}\begin{pmatrix}\text{Asp}\\ \text{Glu}\end{pmatrix}.$$

Table 6 shows the results of the large-scale experiments in which the amino acids present in the peptides were identified by the standard ninhydrin technique. Several preliminary attempts failed to give pure peptides and extensive purification was necessary. Glutamic acid, aspartic acid and glycine were often present as impurities. The spot identified as serine phosphate (band 2) contained more glutamic acid than serine after purification by ionophoresis at pH 3·5 and 6·5.

Since the pentapeptide sequence could be deduced from the composition of the two tripeptides 5 and 6A, special care was taken to obtain these in a pure form, and the results with these two (Table 6) were clear and demonstrate that the pentapeptide sequence is Thr.Ala.SerP.His.Asp. The results with the other peptides are consistent with this sequence, although in most cases they are clearly not completely pure. Thus peptide 7A contained glutamic and aspartic acids in about half the amount of the true components, histidine and serine.

DISCUSSION

In this work we have attempted as far as possible to use methods that depend only on radioactive techniques for the identification of peptides and their degradation products rather than those that depend on the more conventional ninhydrin reaction. Such methods which can be employed on a much smaller scale have the advantage that they can be applied to isotopic peptides that are contaminated with other non-isotopic substances provided they are free from other isotopic substances. The extreme difficulty of purifying peptides from an acid hydrolysate of a large protein is evident from this work, and, if the ninhydrin method had been used by itself, it would have been very difficult to derive the correct sequence. On the other hand, it is relatively easy to obtain isotopic peptides free from one another, and it is hoped that the techniques described here may prove of value in other cases where it is desired to know the sequence around a given labelled amino acid residue.

The method of deduction of the interrelationships of the various peptides from the partial acid hydrolysate (Table 5) depends on the assumption that all the peptide bonds are to some extent hydrolysed by acid. Thus, for instance, peptide 7A was said to be a dipeptide since it gave only serine phosphate on partial hydrolysis. It could possibly have been a tripeptide SerP.X.Y where the bond X–Y was extremely stable so that SerP.X was never produced in appreciable amounts. In fact

Table 7. *Amino acid sequences around reactive serine in enzymes*

Enzyme	Sequence	Reference
Trypsin, Chymotrypsin, Elastase, Thrombin	Gly.Asp.Ser.Gly	Dixon, Kauffman & Neurath (1958); Turba & Gundlach (1955); Schaffer *et al.* (1957); Hartley, Naughton & Sanger (1959); Gladner & Laki (1958)
Liver ali-esterase, Pseudocholinesterase	Gly.Glu.Ser	Jansz, Posthumus & Cohen (1959); Jansz, Brons & Warringa (1959)
Subtilisin	Thr.Ser.Met.Ala	Sanger & Shaw (1960)
Phosphorylase A	Lys.Glu-NH_2.Ileu.SerP.Val.Arg	Fisher, Graves, Grittenden & Krebs (1959)
Phosphoglucomutase	Thr.Ala.SerP.His.Asp	This paper

the further confirmatory experiments did show that all the deductions made from the partial hydrolysis experiments were correct, and whether or not such methods will be entirely reliable can only be decided by further experience. From the partial acid hydrolysis the relationships of the peptides could have been as shown or in the reverse order. The Edman degradation studies showed which was the correct order and confirmed some of the relationships.

The pH–mobility curves have proved useful for identifying charged residues in peptides. It was hoped that they could be used further, for instance to obtain information about the exact pK values of amino and carboxyl groups and about the size of residues involved in peptides. However, preliminary experiments with known serine phosphate peptides and other neutral peptides suggested that accurate results could not easily be obtained. It appeared that other factors, such as adsorption on the paper and hydration, may determine the rate of migration at different pH values, since many of the pH–mobility curves obtained did not agree with any predicted formula (e.g. Alberty, 1953). Thus for instance at all pH values the difference in mobility between SerP.Gly and SerP.Ala was considerably greater than would be expected. At high pH values the migration of serine phosphate peptides was frequently slower than expected, suggesting increased hydration with the increased charge. Perhaps by a more intensive study of the relationships of the structures of peptides to their ionophoretic mobilities, it may be possible to devise methods for identifying residues in peptides in this way. However, at present it seems that more reliable information can be obtained by the use of specific reactions, such as the periodate and the photo-oxidation reaction, and by studying the R_F values of the peptides as described above. With the two chromatographic systems we have used, it was not possible to identify with certainty all the amino acids present in the peptides, though a large number of possibilities could be eliminated. In order to make such a method completely reliable, it would be necessary to use more chromatographic systems and to have more known peptides to use as markers. The formula of Pardee (1951) is not completely accurate and certain peptides may be exceptional (Moore & Baker, 1958). It is to be hoped that with further experience it may be possible to improve the reliability and to develop it as a general method of identifying the amino acid sequence around a given labelled amino acid residue.

The results reported here demonstrate that the structure around the serine phosphate residue is Thr.Ala.SerP.His.Asp (or Asp-NH_2). There seems little doubt that the serine phosphate is involved in the 'active centre' of the enzyme since the phosphate can be exchanged with phosphate in the substrates and, also, since the inactive enzyme does not become labelled. Whether or not the other residues in this sequence are concerned in the enzymic activity cannot be said; but the presence of a histidine residue next to the serine phosphate is of particular interest, since there is some evidence that such a residue is involved in the activity (Koshland *et al.* 1958). There are only three histidine residues in PGM, but whether in fact this particular one is involved in the activity cannot be said with certainty at present.

It is clear that the amino acid sequence around the serine phosphate residue of PGM is in no way similar to that found around the reactive serine residue in other enzymes which react with di*iso*propyl phosphorofluoridate. Some sequences in the immediate vicinity of reactive serine or serine phosphate residues in various enzymes are listed in Table 7. Although there are similarities between different enzymes as between trypsin and chymotrypsin or between liver ali-esterase and pseudocholinesterase, these similarities are not as universal as has sometimes been suggested and cannot at present be made the basis of any general hypothesis.

SUMMARY

1. Phosphoglucomutase labelled with ^{32}P was prepared by two methods involving reaction of the enzyme with labelled substrates.

2. The [^{32}P]phosphoglucomutase was subjected to partial hydrolysis with trypsin and with acid and the [^{32}P]peptides were purified by ionophoresis.

3. The interrelationships of the peptides in the acid hydrolysate were determined by subjecting them to further partial acid hydrolysis and to the Edman degradation procedure.

4. The individual residues in these peptides were identified from the ionophoretic and chromatographic mobilities of the peptides, by specific reactions and from the amino acid composition of highly purified peptides.

5. It was concluded that the amino acid sequence around the serine phosphate (SerP) residue was Thr.Ala.SerP.His.Asp (or Asp-NH_2).

We are grateful to Dr G. Fölsch for a gift of Leu.SerP and Gly.SerP and to Miss R. M. Carpenter for the mitochondria and hexokinase. One of us (C.M.) wishes to acknowledge a scholarship from the British Council.

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A Native Cobalamin–Polypeptide Complex from Liver: Amino Acid Composition and Terminal Amino Acid Analyses of the Peptide Part

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The isolation of a cobalamin–polypeptide from bovine liver was described by Hedbom (1955, 1960). In view of the current interest in protein or peptide conjugates of corrinoids for basic metabolic studies (Wagle, Mehta & Johnson, 1958*a*, *b*; Helleiner & Woods, 1956; Barker, Weissbach & Smyth, 1958) and for the oral treatment of pernicious anaemia (cf. Heathcote & Mooney, 1958) it has seemed desirable to investigate this complex in more detail. Knowledge of its composition and structure might form a basis for general conclusions regarding relationships between structure and biological activity of the corrinoids. The present paper deals with the quantitative amino acid analysis and the determination of the terminal amino acids of the peptide part of the complex.

All results obtained indicate the following composition [for abbreviations see *Biochem. J.* (1957), **66**, 6]: Gly_{22}; Ala_9; Thr_8; Val_4; Leu_{10}; $Ileu_1$; Asp_4; Glu_5; Tyr_4; His_6; $(CySH)_2$; Pro_3; Lys_2, with histidine and glycine as the *N*- and *C*-terminal amino acids respectively.

EXPERIMENTAL

Materials

The samples of cobalamin–peptide used for this work (lots no. 3, 4, 6, 7 and 10) were prepared from ox liver by the method of isolation previously published (Hedbom, 1960). The purified material appeared as a monodisperse substance in the ultracentrifuge. On paper electrophoresis (in citrate, acetate, phosphate and veronal–acetate buffers), within the pH range 2·0–8·6 over which the complex is stable, it migrated as a single entity. Elementary analysis of lot no. 3 after drying gave: C, 52·9; H, 6·7; N, 16·6; S, 0·71; P, 0·342; Co, 0·646%. The moisture content of this was 2·46% as determined by drying *in vacuo* for 24 hr. over P_2O_5 at 100°. Authentic reference samples of dinitrophenyl amino acids were prepared synthetically by Dr H. Kiessling and were kindly supplied by him.

Methods

Isolation of the peptide part. In order to split the cobalamin complex into a corrinoid and a polypeptide part, pure samples of 5–10 mg. were dissolved in 5 ml. of water containing 1 drop of a 10% KCN solution. This process is known to cause a change in the light-absorption so as to give a spectrum resembling that of cyanocobalamin. The shift may be explained by the assumption that a cyanide group has replaced the peptide ligand of the original complex and thus formed a cyanocobalamin and a free peptide.

Preliminary examination by paper electrophoresis at pH 4·5 showed, however, that the cyanocobalamin was still attached to the peptide to a considerable degree by some weak forces of attraction.

Upon slow addition of 1 ml. of 10% trichloroacetic acid the polypeptide precipitated, while the cyanocobalamin remained in solution. After centrifuging and washing of the sediment with diethyl ether the polypeptide was isolated from the last traces of cyanocobalamin by column electrophoresis according to Porath (1956). A volatile buffer of 0·5M-pyridine and 0·1M-acetic acid, pH 4·5, was used. Under these conditions the peptide migrated towards the cathode as a single substance leaving the neutral cyanocobalamin at the starting position. After freeze-drying, the polypeptide was obtained as a white homogeneous powder.

Hydrolysis of the polypeptide. Accurately weighed samples of 5–6 mg. were hydrolysed for 20 and 70 hr. in sealed Pyrex tybes at 105–107°, each with 1 ml. of constant-boiling (5·7N) HCl, which had been redistilled three times in glass. The hydrolysates, which were quite colourless, were processed in the manner described by Smith & Stockell (1954), which involved repeated concentration *in vacuo* at 40–50° followed by dilution with water. The N contents were determined by duplicate micro-Kjeldahl analyses on portions of the hydrolysates.

Amino acid determinations. Initially the dinitrophenylation method developed by Levy (1954) was employed for the amino acid analysis. This method, which is based on paper-electrophoretic separation of dinitrophenyl (DNP) amino acids, gave poor results in our hands. For this reason the column-chromatographic technique of Moore & Stein (1951) had to be used for the accurate determination. It was later possible to confirm the reproducibility of this determination by use of the Spinco automatic amino acid analyser, when this apparatus became available.

Dinitrophenylation method. The quantitative dinitrophenylation was carried out both on 20 and 70 hr. hydrolysates with a slight excess of 1-fluoro-2:4-dinitrobenzene (FDNB) at pH 9·0 and 40°. Excess of reagent was removed by sublimation according to the method described by Mills (1952). The separation of the DNP amino acids was effected by two-dimensional chromatography on Whatman no. 4 paper with toluene–chloroethanol–pyridine–aq. 0·8N-NH_3 soln. (Biserte & Osteux, 1951) or with 2-methylbutan-2-ol (Blackburn & Lowter, 1951) in the first dimension and with aq. 1·5M-phosphate buffer in the second. The DNP derivatives were identified by comparison with known DNP amino acids. For quantitative estimation, the coloured spots were cut out together with appropriate blanks, and eluted by shaking for 15 min. at 60° in 4 ml. portions of aq. 1% (w/v) Na_2CO_3. The extinctions of the extracts were read against the blank extract at the maxima near 360 mμ (385 mμ for DNP-proline) in a Beckman DU spectrophotometer. These readings were converted into μmoles of DNP amino acids as described by Levy (1954) with the same conversion factors. Duplicate analyses were performed for each hydrolysate and each analysis was carried out with three different chromatograms.

Column chromatography. For the more accurate amino acid analyses the method described in detail by Moore, Spackman & Stein (1958) and Spackman, Stein & Moore (1958) was used. The separation and determination of the amino acids were performed in a Beckman/Spinco model MS amino acid analyser. The columns were packed with a sulphonated styrene, 8%, divinylbenzene co-polymer, labelled 'Aminex-MS cation-exchange resin', obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. The resin was graded according to the method of Hamilton (1958). A particle size of 31–45 μ was used for the 150 cm. column and 19–25 μ for the 15 cm. column.

Oxidation with performic acid. To cleave possible disulphide bridges in the molecule, a polypeptide sample of 5 mg. dissolved in 1 ml. of 85% formic acid was oxidized by the addition of 0·1 ml. of 30% H_2O_2. The reaction was allowed to proceed for 30 min. at room temperature (22°) after which the solution was diluted with 10 ml. of ice–water. The excess of reagent was removed by freeze-drying. The residue was redissolved in 2 ml. of water and evaporated to dryness *in vacuo*.

N-Terminal analysis. The polypeptide was dinitrophenylated by shaking amounts of 5–6 mg. with excess of FDNB in 67% ethanol containing $NaHCO_3$ or trimethylamine; essentially the method of Sanger (1945) was used. Excess of reagent was removed by extraction with ether, after acidifying the product with HCl. The precipitated yellow DNP peptide was then hydrolysed with 5·7N-HCl for 8 or 20 hr. After removal *in vacuo* of the excess of HCl, the yellow residue was dissolved in 0·5N-HCl and extracted four times with diethyl ether. The ether extracts and the

water phase were evaporated to dryness, and the residues dissolved in water and used for paper-chromatographic analysis of the DNP amino acids, as described above.

C-Terminal analysis. Some preliminary attempts were made to identify the *C*-terminal amino acids by reduction of the free carboxylic groups with $LiAlH_4$. Hydrolysis of the reaction product gave a mixture of amino acids and amino alcohols which was investigated by paper chromatography.

In a typical experiment 8 mg. of polypeptide was dried *in vacuo* for 24 hr. at 100° over P_2O_5. The sample was suspended in 10 ml. of dry ether and an ether solution of 100 mg. of $LiAlH_4$ was slowly added. The reaction was allowed to proceed for 10 hr. during which time the mixture was stirred continuously. The reaction mixture was then neutralized with HCl and evaporated to dryness. The residue, containing the reduced polypeptide, was hydrolysed for 20 hr. in 5·7 N-HCl and analysed for amino acids and amino alcohols by paper chromatography as described by Hedbom (1960).

RESULTS

Amino acid composition. The dinitrophenylation method furnished preliminary data regarding the amino acid composition. DNP-leucine and DNP-isoleucine were not separated, and sometimes DNP-glutamic acid and DNP-aspartic acid were poorly resolved, but otherwise the separation was satisfactory. The results of the amino acid analysis by the Levy method is reported in Table 1. The amounts of glycine, leucine, glutamic acid, tyrosine and proline are low compared with the values obtained by the Moore & Stein procedure (see below). As mentioned in the Discussion, a possible reason for this may be that the factors for the conversion of the extinctions into molar ratios, given by Levy for insulin, are not completely applicable to other polypeptides. It would appear that the following factors, which are on average about 6 % higher than those given by Levy, are more nearly correct for the cobalamin–polypeptide: Asp 1·04; Thr 1·12; Glu 1·15; Pro 1·04; Gly 1·11; Ala 1·08; Val 0·94; Leu 1·15; Tyr 1·96; Lys 0·69; His 1·88; CySH 1·12.

Control experiments with a commercial amino acid calibration mixture (Spinco 120–220 lot 191131) also indicated that the Levy factors were 5–8 % too low. Here also, the chromatographic resolution of the coloured spots was satisfactory, and the blank readings were very low (less than 0·001/cm.²). For these reasons it would seem that the discrepancy is significant.

Ion-exchange chromatography gave satisfactory results for the amino acid analysis. Some features of the composition are illustrated qualitatively in the elution curve of Fig. 1, obtained with a sample hydrolysed for 20 hr. From these data it is apparent that the polypeptide has a relatively high content of glycine and that serine, arginine, phenylalanine and methionine are not present. The absence of tryptophan has previously been shown by spectroscopic examination of an unhydrolysed sample of the polypeptide. No evidence was found suggesting incompleteness of the hydrolysis. The base-line showed no significant variations, and the peaks were entirely symmetrical, and were apparently not contaminated with residual peptides. Tables 2 and 3 summarize the data from the experiments with 20 and 70 hr. hydrolysates of two samples of the polypeptide. In instances where there was evidence of destruction, as with aspartic acid, threonine, cystine and tyrosine, corrections for the losses were calculated by extrapolating the analytical data to zero time, assuming first-order kinetics for the decomposition. The value which

Table 1. *Amino acid analysis of the cobalamin–polypeptide by the dinitrophenylation method*

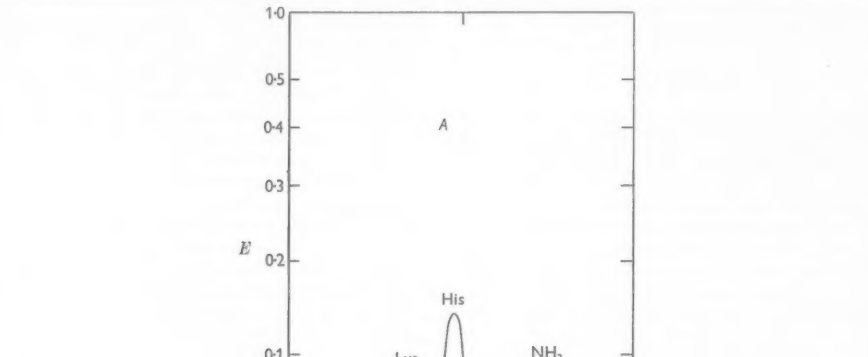
Samples were from preps. 3 and 4. Average quantity found is the average of duplicate analyses of 20 and 70 hr. hydrolysates. The variation is expressed as the average deviation. The calculated no. of amino acid residues are on a basis of a mol.wt. of 7900 from the cobalt content (Hedbom, 1960).

Amino acid	Average quantity found (μmoles)	Calculated no. of amino acid residues	Residues taken to nearest integer
Asp	0·482 ± 0·014	3·81	4
Thr	1·03 ± 0·035	8·11	8
Glu	0·530 ± 0·012	4·19	4
Pro	0·224 ± 0·011	1·77	2
Gly	2·58 ± 0·070	20·4	20
Ala	1·29 ± 0·034	9·2	9
CySH	0·366 ± 0·019	2·89	3
Val	0·535 ± 0·014	4·23	4
Leu + Ileu	1·32 ± 0·040	10·4	10
Tyr	0·406 ± 0·027	3·21	3
Lys	0·232 ± 0·013	1·85	2
His	0·645 ± 0·024	5·11	5
		Total	74

may be subject to the greatest error is that for cystine because of its instability to hydrolysis. The calculated number of two cysteine residues per molecule is in good agreement with the sulphur content. Experiments with performic acid-oxidized samples also support this value. Paper-electrophoretic examination of such samples at pH values of 3·0, 4·5 and 8·0 showed that the peptide still migrated as one homogeneous substance. No evidence for the degradation of the peptide to smaller fragments was found. At pH 4·5 the untreated peptide migrated towards the cathode, whereas the oxidized peptide under the same conditions slowly moved in the opposite direction, with a mobility that could be explained by the presence of about two additional negatively charged groups in the molecule.

The foregoing results account for the entire composition of the polypeptide. The recovery of amino acids was in accordance with the total weight, and the nitrogen content was within the limits of the experimental method used.

End groups. No ether-soluble DNP amino acids were obtained from the 20 hr. hydrolysate of the polypeptide nor from a sample that had been hydrolysed for 8 hr. Coloured material in the ether phase proved to be dinitrophenol, dinitroaniline and small amounts of the water-soluble DNP derivatives. Increased time for the reaction with FDNB failed to produce any ether-soluble DNP amino acids.



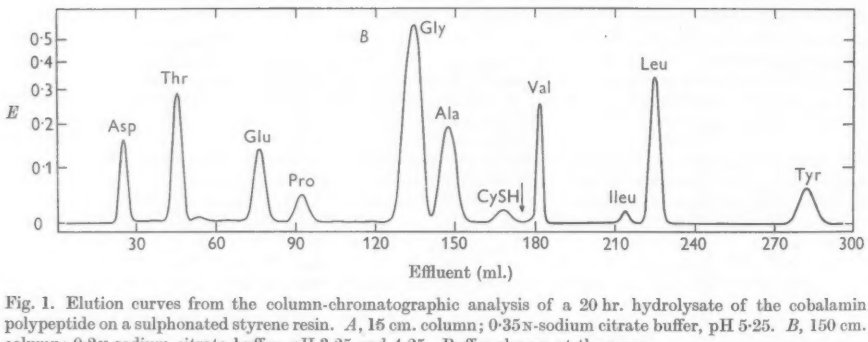


Fig. 1. Elution curves from the column-chromatographic analysis of a 20 hr. hydrolysate of the cobalamin polypeptide on a sulphonated styrene resin. *A*, 15 cm. column; 0·35 N-sodium citrate buffer, pH 5·25. *B*, 150 cm. column; 0·2 N-sodium citrate buffer, pH 3·25 and 4·25. Buffer change at the arrow.

The water phase contained ε-DNP-lysine, bis-DNP-histidine, imino-DNP-histidine and minute amounts of α-DNP-histidine. The dinitrophenylation seemed to be complete since no free lysine could be detected in the hydrolysate.

Calculations from the ultraviolet spectra of the eluted bis-DNP-histidine spots gave an average recovery of 0·15 mg. of histidine/8 mg. of polypeptide, i.e. 0·92 mole of terminal histidine/mole of polypeptide. These data show that histidine is the *N*-terminal amino acid.

After reduction with $LiAlH_4$ and hydrolysis, the sample, when subjected to paper chromatography, gave two additional ninhydrin-positive spots compared with the hydrolysate of the untreated peptide. Only one of these could be identified with reasonable confidence as an amino alcohol. This substance had the same R_F as ethanolamine obtained from a control sample of leucylglycine reduced and hydrolysed in the same way. Although no reliable quantitative estimation could be made from the chromatograms it seems likely from the data obtained that glycine constitutes the *C*-terminal group.

Table 2. *Amino acid analysis of the cobalamin–polypeptide by column chromatography*

The values given for the 20 and 70 hr. hydrolysates represent duplicate analyses of samples from two different preparations.

	Amino acid residue (g./100 g. of polypeptide)										
	Time of hydrolysis, 20 hr.					Time of hydrolysis, 70 hr.					
Amino acid	Sample from prepn. 7		Sample from prepn. 10		Av.	Sample from prepn. 7		Sample from prepn. 10		Av.	Av. or extrapolated value
Asp	5·73	5·67	5·81	5·79	5·75	5·33	5·28	5·37	5·40	5·35	5·92*
Thr	10·16	10·26	10·14	10·16	10·18	9·63	9·57	9·69	9·67	9·64	10·42*
Glu	8·54	8·56	8·62	8·60	8·58	8·46	8·42	8·54	8·50	8·48	8·53
Pro	3·76	3·70	3·76	3·74	3·74	3·68	3·74	3·72	3·76	3·71	3·73
Gly	16·18	15·85	16·51	16·29	16·21	15·86	15·98	15·95	15·83	15·91	16·06
Ala	8·30	8·24	8·25	8·17	8·24	8·64	8·74	8·46	8·64	8·62	8·43
CySH†	2·36	2·43	2·52	2·60	2·48	2·08	2·16	2·07	2·09	2·10	2·64*
Val	5·16	5·30	5·14	5·16	5·19	5·16	5·10	5·10	5·24	5·15	5·17
Ileu	1·54	1·54	1·48	1·44	1·50	1·42	1·38	1·45	1·42	1·42	1·46
Leu	15·26	15·24	15·21	15·09	15·20	14·48	14·60	14·61	14·39	14·52	14·86
Tyr	8·40	8·49	8·55	8·57	8·46	8·16	8·19	8·20	8·23	8·20	8·57*
Lys	3·52	3·47	3·47	3·33	3·45	3·24	3·15	3·34	3·18	3·23	3·34
His	10·54	10·46	10·40	10·46	10·43	10·71	10·69	10·68	10·56	10·66	10·55
Amino (NH_2)	0·35	0·41	0·44	0·36	0·39	0·39	0·49	0·47	0·45	0·45	0·42

* Extrapolated value. † Measured as cystine but calculated as cysteine.

Table 3. *Amino acid composition of the cobalamin polypeptide*

The result is in agreement with the following structure: Gly_{22}; Ala_9; Thr_8; Val_4; Leu_{10}; $Ileu_1$; Asp_4; Glu_5; Tyr_4; His_6; $(CySH)_2$; Pro_3; Lys_2.

Amino acid	Amino acid residue (g./100 g. of polypeptide)	Min. mol.wt.	Mol.wt.	Amino acid (g./100 g. of polypeptide)	N (% of total N)	Calculated no. of residues for mol.wt. 7712
Asp	5·92	1944	7776	6·84	4·21	3·97
Thr	10·42	970·2	7762	12·27	8·44	7·95
Glu	8·53	1513	7565	9·72	5·41	5·10
Pro	3·73	2604	7812	4·42	3·15	2·96
Gly	16·06	355·2	7814	21·13	23·06	21·70
Ala	8·43	843·2	7589	10·56	9·71	9·15
CySH	2·64	3905	7810	3·10	2·11	1·97
Val	5·17	1917	7668	6·11	4·27	4·02
Ileu	1·46	7753	7753	1·69	1·05	0·99
Leu	14·86	761·8	7618	17·22	10·75	10·10
Tyr	8·57	1904	7616	9·51	4·30	4·05
Lys	3·34	3838	7676	3·81	4·27	2·01
His	10·55	1300	7800	11·93	18·89	5·93
Amino (NH_2)	0·42	4448	(8896)*	—	2·02	(1·73)*
Total	100·10	—	—	118·31	101·64	79·90
Mean	—	—	7712	—	—	—

* Omitted value.

DISCUSSION

Evidence for the homogeneity of the isolated cobalamin–polypeptide complex has been put forward earlier (Hedbom, 1960). The results reported in the present paper substantiate these findings. The general amino acid composition and the terminal amino acids are shown to be the same within the limits of experimental error, in four or five samples of peptide from different preparations. This was determined by dinitrophenylation in three cases, and by column chromatography in two. Concerning the dinitrophenylation method for the determination of the amino acid composition, Li & Chung (1956) reported excellent results from analyses of a hypophyseal growth hormone with a molecular weight of 45 000 and of bovine plasma albumin of 65 000. As the cobalamin–polypeptide has a molecular weight of less than 10 000, we had hoped that the method would give the exact and correct number of residues. Direct calculations from the extinction values of the eluated DNP amino acids gave molar ratios corresponding to only 75 residues. It is possible, however, to deduce the number of residues from the alkali uptake during the dinitrophenylation reaction. This was not done, but if the correct value of 80 residues (from Table 3) is used for the calculation the following composition is obtained: Asp 4·07; Thr 8·60; Glu 4·47; Pro 1·90; Gly 22·20; Ala 9·80; CySH 3·10; Val 4·50; Leu+Ileu 11·1; Tyr 3·44; Lys 1·95; His 5·47.

If these numbers are taken to the nearest integer the result is (the correct number, if different, is given in parentheses): Asp 4; Thr 9 (8); Glu 4 (5); Pro 2 (3); Gly 22; Ala 10 (9); CySH 3 (2); Val 4 or 5 (4); Leu+Ileu 11; Tyr 3 (4); Lys 2; His 5 (6).

The accuracy of the determination of the molar fractions was within ±3%, and we must therefore conclude that the Levy method was not sufficiently precise to give the correct amino acid composition of the polypeptide. A simple, possible explanation would be that the dinitrophenylation reaction was not quantitative. The method has, however, some advantages of speed and sensitivity; for the present work it was of value for determining the approximate amino acid composition of the polypeptide, and later for analysing smaller degradation peptides of the cobalamin complex.

The accurate amino acid analysis, as reported in Tables 2 and 3, showed that the peptide part of the complex contained 80 residues: Gly_{22}; Ala_9; Thr_8; Val_4; Leu_{10}; $Ileu_1$; Asp_4; Glu_5; Tyr_4; His_6; $(CySH)_2$; Pro_3; Lys_2; with a calculated molecular weight of 7698. In the analysis 100·10% of the total weight and 101·64% of the nitrogen content was accounted for. The calculated molecular weight corresponds to 9028 for the whole complex. This is in good agreement with the earlier reported figure of 9129 from the cobalt content and 9053 from absorption spectroscopy. That this number represents the true molecular weight rather than a multiple of it is supported by results of centrifuging (Hedbom, 1960).

The polypeptide has a single *N*-terminal amino acid, as judged from the finding of 0·92 mole of histidine as DNP derivative. The identification of ethanolamine in the reduced and hydrolysed peptide sample indicated glycine to be one *C*-terminal group. Degradation with carboxypeptidase gave glycine as the only *C*-terminal (A. Hedbom & R. Mosbach, unpublished observations). Treatment with performic acid to break possible S·S bonds did not result in the rupture of the peptide chain into smaller fragments. This evidence suggests a straight-chain gross structure of the polypeptide, possibly with a ring formed by a cross-linked cystine residue.

A close examination of the amino acid distribution reveals the presence of four aspartic acid, five glutamic acid and eight basic residues, i.e. six histidine and two lysine. It is thus likely that the isoelectric point of the peptide should be near a pH where the imidazole groups of histidine are in the cationic form. The measured isoelectric point of 6·25 is in good agreement with the analytical findings.

The glycine content is remarkably high; 22 residues of the total of 80 represents 16·27% of the total weight.

SUMMARY

1. The cobalamin–polypeptide from ox liver has been analysed for amino acids by the dinitrophenylation method and by ion-exchange chromatography. The terminal amino acids have been determined.

2. The dinitrophenylation method was inadequate for amino acid analysis of the peptide. The reasons for this are discussed.

3. The polypeptide has been shown to consist of a single chain of 80 amino acid residues, possibly with a ring formed by a cross-linked cystine residue.

4. The composition of the polypeptide is consistent with the following formula: Gly_{22}; Ala_9; Thr_8; Val_4; Leu_{10}; $Ileu_1$; Asp_4; Glu_5; Tyr_4; His_6; $(CySH)_2$; Pro_3; Lys 2. Glycine and histidine are the *C*- and *N*-terminal residues respectively.

5. The reported composition is in good agreement with the molecular weight and the nitrogen content of the polypeptide part as well as of the whole cobalamin complex.

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The Mechanism of the Glyoxalase I Reaction, and the Effect of Ophthalmic Acid as an Inhibitor

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The glyoxalase system catalyses the two reactions:

Methylglyoxal + glutathione → *S*-lactoylglutathione → lactic acid + glutathione

and each reaction is catalysed by a separate enzyme, glyoxalases I and II respectively (Racker, 1951; Crook & Law, 1952). The work described is concerned with glyoxalase I only, which, for brevity, will be referred to simply as glyoxalase. The thiol group of glutathione plays a part in the reaction, and ophthalmic acid, a tripeptide, isolated from calf lens, which is structurally similar to glutathione but lacks the thiol group (Waley, 1958), might be expected to inhibit the reaction. This has been demonstrated. Synthetic analogues of glutathione are known to act as inhibitors in the glyoxalase reaction (Kermack & Matheson, 1957).

In an enzymic reaction in which there are two substrates, it is possible that the two substrates might react with each other before reacting with the enzyme. In general, this does not happen. The evidence presented here, however, suggests that this occurs with the glyoxalase reaction. In that case, the intermediates in the complete glyoxalase system will be as set out in Scheme I.

A preliminary account of this work has already appeared (Cliffe & Waley, 1959).

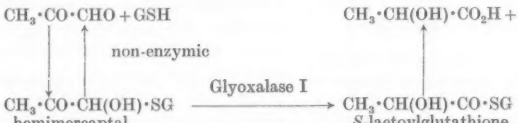
EXPERIMENTAL

Materials

Methylglyoxal was purified and standardized as described by Kermack & Matheson (1957), and the glutathione (GSH) was obtained from The Distillers Co. Ltd. (Biochemicals). Ophthalmic acid and norophthalmic acid were synthetic samples (Waley, 1957, 1958). We are indebted to Dr K. W. Daisley for the preparation of glyoxalase I, which had been purified to step 4 in the procedure of Racker (1951), and was free from glyoxalase II.

Methods

The formation of *S*-lactoylglutathione was followed by the increase in $E_{240\ m\mu}$ in a Beckman DU spectrophotometer (Racker, 1951); optical cells with 1 cm. light-path were used. The reactions were carried out in 33 mM-potassium



Scheme I

phosphate buffer pH 6·6, at room temperature which was held at 20±1°. Readings were taken against a blank cell containing water and buffer. Each day a portion of the stock glyoxalase I preparation was diluted with 0·1 M-potassium phosphate buffer, pH 7·4, that contained crystalline bovine serum albumin (100μg./ml.); 0·1 ml. of the diluted enzyme solution was used in each reaction mixture, whose total volume was 3 ml. As the extent of dilution of the stock solution of glyoxalase I (usually 200- to 500-fold) varied in different experiments, unit concentration of the enzyme is taken as 0·1 ml. of undiluted stock solution in 3 ml. Solutions of GSH were prepared immediately before use, and the pH was brought to 6·6 with aqueous potassium hydroxide.

Except where otherwise stated, the substrates were mixed and, 10 min. later, the solution of the enzyme was added; this ensured that the equilibrium concentration of the hemimercaptal had been attained before the enzymic reaction started. The molar extinction coefficients of the hemimercaptal and of *S*-lactoylglutathione are 220 and 3300 respectively.

RESULTS

Effect of substrate concentrations on the rate of the reaction

The effect of variation of the concentrations of methylglyoxal and of GSH on the rate of formation of *S*-lactoylglutathione is shown in Fig. 1. Each curve represents the results of two series of experiments. The lower curve shows one series of points for a fixed concentration (1 mM) of methylglyoxal and variable GSH concentration, and another series of points for a fixed concentration (1 mM) of GSH and variable methylglyoxal concentration. The upper curve is similar, except that the value of the fixed concentration was 2 mM. One feature is immediately obvious: within the experimental error, all the points for a given series lie on

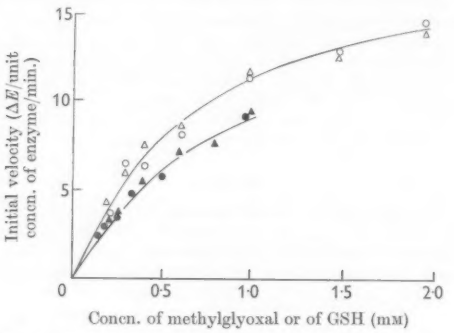


Fig. 1. Variation of initial rate of formation of *S*-lactoylglutathione with concentration of methylglyoxal when the concentration of GSH is 1 mM (●), or 2 mM (○), and with concentration of GSH when the concentration of methylglyoxal is 1 mM (▲) or 2 mM (△).

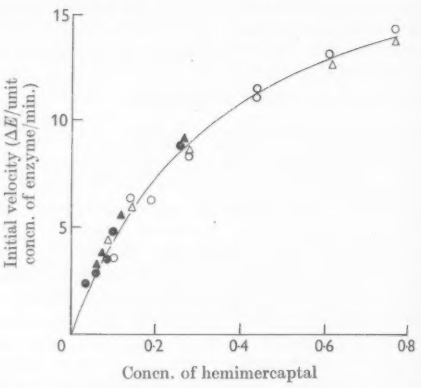


Fig. 2. Variation of initial rate of formation of *S*-lactoylglutathione with the calculated concentration of hemimercaptal. Each point corresponds to a point in Fig. 1, and the same symbols have been used. The concentrations of the hemimercaptal have been obtained from those of methylglyoxal and GSH; the dissociation constant of the hemimercaptal has been taken as 2 mM.

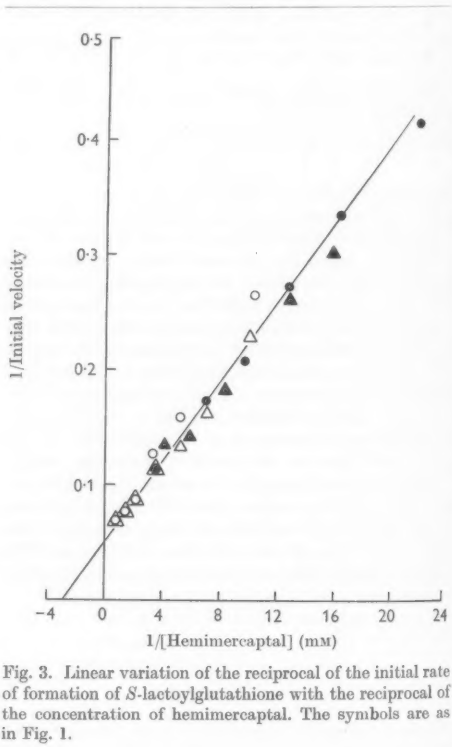


Fig. 3. Linear variation of the reciprocal of the initial rate of formation of *S*-lactoylglutathione with the reciprocal of the concentration of hemimercaptal. The symbols are as in Fig. 1.

the same curve. For example, the rate is the same when the concentration of methylglyoxal is 2 mM and that of GSH 1 mM as it is when the concentration of methylglyoxal is 1 mM and that of GSH 2 mM. It is clear that the velocity is a symmetrical function of the concentrations of methylglyoxal and of GSH. This is the test suggested by Segal, Kachmar & Boyer (1952) for a mechanism in which the two species, here methylglyoxal (M) and glutathione (G), unite to form an adduct (Q), which then combines with the enzyme (E):

$$M + G \rightleftarrows Q \qquad (1)$$
$$Q + E \rightleftarrows QE \rightarrow \text{product} + E$$

Now if the adduct (Q) is really the substrate (which is what this mechanism amounts to) then, when the velocity is plotted against the concentration of adduct, all the points should now lie on one curve. That this is so is shown in Fig. 2. The same feature is also seen in the linear plot obtained when the reciprocal of the velocity is plotted against the reciprocal of the concentration of the adduct (Fig. 3): all the points lie on one straight line. Statistically, there is no significant difference between the gradients and intercepts of the best straight lines for each four series of experiments.

To obtain the concentration of the adduct (Q) the equilibrium constant (K) of reaction (1) has to be known. This was determined by the method of Kermack & Matheson (1957) from measurements of the increase in E when solutions of methylglyoxal and of GSH are mixed. Our value (2 mM) for this equilibrium constant differs from theirs (5 mM), but the method is rather inaccurate, and the differing values are not important since the shape of the curve is not much affected by the exact value chosen for the calculated concentration of adduct.

This evidence, then, suggests that the formation of the adduct from methylglyoxal and GSH is a preliminary to the enzymic reaction proper. The mechanism of the formation of the adduct has therefore been investigated.

Mechanism of the formation of hemimercaptal

The rate of formation of the adduct was followed by the increase in $E_{240\ m\mu}$; the conditions of pH concentration, of buffer and temperature were the same as in the enzymic experiments. When the initial concentrations of methylglyoxal and of GSH were independently varied, the results (Table 1) showed that the initial rate was independent of the concentration of GSH, but linear with respect to the concentration of methylglyoxal. The reaction was rather rapid and so the initial rate could not be accurately measured, and the extent of reaction after a fixed interval of time (Table 2) is more suitable as a test of the kinetic scheme given below. A one-step bimolecular mechanism [reaction (1)], which requires that the extent of reaction should depend on the concentrations of both partners, is therefore excluded.

Since the rate-determining step involves only methylglyoxal, the simplest kinetic scheme is one in which the methylglyoxal (M) is converted into a more reactive species (M*) (the chemical nature of this change is discussed below), which then reacts with GSH (G) to give the hemimercaptal

$$[CH_3 \cdot CO \cdot CH(OH) \cdot SG]\ (Q):$$

$$M \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} M^*$$

$$M^* + G \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} Q$$

where k_1, k_{-1}, k_2 and k_{-2} are the appropriate velocity constants. The rate of change of the concentration of M* is then

$$dm^*/dt = k_1 m - k_{-1} m^* - k_2 m^* g + k_{-2} q$$

where m^*, m, g and q denote respectively the concentrations of the species M*, M, G and Q in the mechanism written above. If M* is a reactive

Table 1. *Initial rate of formation of hemimercaptal*

The rates are measured by increase in $E_{240\ m\mu}$ from methylglyoxal and glutathione and are given as ΔE/sec.

Concn. of GSH (mM)	Concn. of methylglyoxal (mM)				
	1	2	3	4	5
1	0·0018	—	—	—	—
2	0·0019	0·0036	0·0056	—	—
3	0·0018	0·0039	—	0·0082	0·0100
4	0·0020	—	—	—	0·0090
5	0·0020	—	0·0057	—	0·0092

Table 2. *Extent of formation of hemimercaptal from methylglyoxal and glutathione after a fixed interval of time, and the first-order rate constant (k_1) obtained by the use of equation* (3) *(see text)*

Concn. of methylglyoxal (mM)	Concn. of GSH (mM)	$\Delta E_{240\ m\mu}$ after 37 sec.	Concn. of hemimercaptal formed (mM)	$10^3\ k_1$ (sec.$^{-1}$)
1	1	0·062	0·281	8·9
1	2	0·070	0·317	10·2
1	3	0·063	0·285	9·1
1	4	0·065	0·294	9·5
1	5	0·075	0·339	11·1
2	2	0·138	0·624	10·1
2	4	0·145	0·591	9·5
3	3	0·195	0·882	9·5
3	5	0·252	1·14	12·8
4	4	0·313	1·42	11·8
5	3	0·348	1·57	10·2
5	4	0·340	1·54	9·9
5	5	0·330	1·49	9·5

intermediate, so that the stationary state approximation may be applied,

$dm^*/dt = 0$, and $m^* = (k_1 m + k_{-2} q)/(k_{-1} + k_2 g)$.

Thus the rate of formation of the adduct (Q) is given by

$$dq/dt = k_2 m^* g - k_{-2} q,$$

i.e. $dq/dt = k_2 g(k_1 m + k_{-2} q)/(k_{-1} + k_2 g) - k_{-2} q$.

If the reactive intermediate (M*) reacts much more rapidly with GSH than it reverts to M, i.e. if $k_2 g \gg k_{-1}$, then for the early stages of the reaction, when $q \doteq 0$,

$$dq/dt = k_1 m.$$

As $dm^*/dt = 0$, $dq/dt = -dm/dt$, and $m = m_0 - q$, where m_0 is the initial concentration of M, we may write

$$-dm/dt = k_1 m, \quad \text{and} \quad q = m_0 (1 - e^{-k_1 t}). \qquad (2)$$

This equation shows that the amount of adduct (Q) formed after a fixed time (t) is proportional to the initial concentration of methylglyoxal, in agreement with the results in Table 2. Rearrangement of equation (2) gives equation (3), from which, together with the data in Table 2, the value of k_1 was found to be 0·01 sec.$^{-1}$

$$k_1 = (1/t) \ln [m_0/(m_0 - q)]. \qquad (3)$$

This reaction is discussed in more detail later, but we may mention here that the species symbolized by M* is probably free methylglyoxal and M is a hydrate of methylglyoxal. The rate-determining step in the formation of the hemimercaptal is then liberation of free methylglyoxal from its hydrate.

Interplay of the enzymic and non-enzymic reactions

Under suitable conditions, the rate of the non-enzymic reaction may affect the rate of formation of *S*-lactoylglutathione. Two sets of experiments were carried out, each at several enzymic concentrations. The sets differed in the concentrations of GSH and of methylglyoxal: in one set ('high methylglyoxal') the concentration of methylglyoxal was 2 mM and that of GSH 0·1 mM; in the other set ('low methylglyoxal') the concentrations were 0·1 and 2 mM respectively. In these experiments, the methylglyoxal was added last to the other constituents of the reaction mixture. Thus both the enzymic and non-enzymic reactions started at the same time. At the higher concentrations of enzyme the initial rate was too rapid to measure accurately, and the results in Fig. 4 are expressed as the change in E after 1 min. At the lower concentrations of enzyme the two curves are superimposable, and the velocity is a symmetrical function of the substrate concentrations, as already described. At the higher concentrations of enzyme, however, the two curves diverge. The 'high methylglyoxal' curve rises steadily with increase of enzyme concentration, but the 'low methylglyoxal' curve flattens off at a relatively low concentration of enzyme. In the latter, the rate of reaction is controlled by a non-enzymic step, which is (on our interpretation) the formation of the adduct from GSH and methylglyoxal. The rate of

Fig. 4. Variation in rate of reaction (as measured by the ΔE after 1 min.) with the concentration of enzyme. The 'low methylglyoxal' cell (●) contained methylglyoxal (0·1 mM) and GSH (2 mM), and the 'high methylglyoxal' cell (○) contained methylglyoxal (2 mM) and GSH (0·1 mM).

Fig. 5. Inhibition of the glyoxalase reaction by ophthalmic acid. The initial rates were measured for several concentrations of ophthalmic acid at GSH concentrations of 0·1 mM (○), 0·2 mM (△), 0·5 mM (●) and 1·0 mM (□). The concentration of methylglyoxal was 2·37 mM throughout.

formation of the adduct was shown above to depend on the concentration of methylglyoxal. These experiments, then, are consistent with the mechanism in which the adduct is the substrate.

Ophthalmic acid as inhibitor

The initial rates of formation of *S*-lactoylglutathione were measured for given concentrations of GSH and of methylglyoxal and various concentrations of ophthalmic acid; this procedure was repeated for several given concentrations of GSH. The results (Fig. 5) show that ophthalmic acid behaves as a competitive inhibitor; the equilibrium constant for the formation of the inactive enzyme–inhibitor compound, determined by the method of Dixon (1953), is 0·95 mM. Norophthalmic acid was also an inhibitor, but insufficient material was available for a determination of K_i for this compound.

DISCUSSION

Formation of the hemimercaptal from methylglyoxal and glutathione

The nature and composition of the mixture of hydrates and polymers in an aqueous solution of methylglyoxal is not known (Moulds & Riley, 1938). Aldehydes are hydrated (to a varying extent) in solution, and the stability of the hydrate is greater when electron-withdrawing groups are present (as in chloral hydrate). Thus methylglyoxal would be expected to be hydrated in solution. Moreover, α-dicarbonyl compounds are often coloured, and monomeric methylglyoxal is yellow, but the aqueous solution is colourless and has an absorption maximum at about 270 mμ. There is, then, little free methylglyoxal ($CH_3 \cdot CO \cdot CHO$) present in the aqueous solution. The adduct from methylglyoxal and GSH (Schubert, 1935; Yamazoe, 1936) is taken to be the hemimercaptal

$$[CH_3 \cdot CO \cdot CH(OH) \cdot SG]$$

and this seems the most likely structure on chemical grounds. The formation of this hemimercaptal requires free methylglyoxal, since the electrophilic carbon atom of the aldehyde group is absent in the hydrate or polymers derived from it. Hence the first step in the formation of the hemimercaptal is the liberation of free methylglyoxal. This accounts for the results given in Table 1, which show that the initial rate of formation of the hemimercaptal depends on the concentration of methylglyoxal and is independent of the concentration of GSH.

Kermack & Matheson (1957) used the increase in $E_{240\ m\mu}$ as a measure of the extent of hemimercaptal formation. Earlier workers (e.g. Platt & Schroeder, 1934) had reported that from 20 to 80 % of the GSH was not free in acid solutions of GSH and methylglyoxal. Knox (1960) suggested that there was a discrepancy between these figures and the value that Kermack & Matheson obtained for the equilibrium constant. Our value for the equilibrium constant indicates that approximately 30–40 % of the GSH is not free, at pH 6·6. The extent of hemimercaptal formation is known to be greater in more acid solution, and there does not seem to be any large discrepancy between our results and those of earlier workers.

Nature of the substrate in the glyoxalase reaction

The fact that the velocity of the formation of *S*-lactoylglutathione is a symmetrical function of the concentrations of methylglyoxal and GSH (Fig. 1) has been interpreted by the hypothesis that the hemimercaptal is the substrate for glyoxalase. We now have to consider whether other mechanisms could give the same result. Several mechanisms for enzymic reactions that involve two species yield a rate equation of the following type (Dalziel, 1957):

$$\frac{e}{v_0} = \Phi_0 + \frac{\Phi_1}{[S_1]} + \frac{\Phi_2}{[S_2]} + \frac{\Phi_{12}}{[S_1]\,[S_2]}, \qquad (4)$$

where e is the total concentration of the enzyme, v_0 is the initial rate, $[S_1]$ and $[S_2]$ are the initial concentrations of the two substrates, and the four parameters Φ_0, Φ_1, Φ_2, Φ_{12} are functions of the rate constants in the assumed mechanism. Practical considerations limit the extent to which the concentrations of the substrates can be varied, and so the fact that the results fit equation (4) is not, of itself, a satisfactory guide to the mechanism. Now, if the velocity is to be a symmetrical function of S_1 and S_2 it is necessary that

$$\Phi_1 = \Phi_2.$$

The mechanism put forward by Kermack & Matheson (1957) may be written:

$$E + S_1 \rightleftarrows ES_1 \text{ equilibrium constant } K_1$$
$$E + S_2 \rightleftarrows ES_2 \text{ equilibrium constant } K_2$$
$$ES_1 + S_2 \rightleftarrows ES_1S_2 \text{ equilibrium constant } K_3$$
$$ES_2 + S_1 \rightleftarrows ES_1S_2 \text{ equilibrium constant } K_4$$
$$ES_1S_2 \xrightarrow{k} \text{product} + E.$$

They assumed that the rate of formation of product from the ternary complex is small compared with the rates of the other steps; this is the 'equilibrium' assumption (Segal, 1959) which is necessary if the data are to fit equation (4), which now takes the form (Dalziel, 1957):

$$\frac{e}{v_0} = \frac{1}{k}\left[1 + \frac{K_4}{[S_1]} + \frac{K_3}{[S_2]} + \frac{K_1K_3}{[S_1]\,[S_2]}\right]. \qquad (5)$$

Kermack & Matheson also suggested that combination of the enzyme with one substrate did not affect its affinity for the other, i.e. that $K_1 = K_4$

and $K_2 = K_3$. For the velocity to be a symmetrical function of the concentrations of the two substrates it is thus necessary that $K_3 = K_4$, so that all the equilibrium constants have a common value (K'), i.e. the enzyme has the same affinity for both substrates, and equation (5) becomes

$$\frac{e}{v_0} = \frac{1}{k}\left[\left(1+\frac{K'}{[S_1]}\right)\left(1+\frac{K'}{[S_2]}\right)\right]. \qquad (6)$$

The fit of the data to equation (6) has been tested as follows. The results plotted in Fig. 4 of Kermack & Matheson's (1957) paper gave a value of 0·46 mM for K' [other mechanisms would also give the same value for this equilibrium constant (Frieden, 1957)]. This value of K' may be used to obtain a value of the rate constant, k, for the series of experiments (Kermack & Matheson, 1957) in which the concentration of methylglyoxal was 1·08 mM. The values of k and K' may then be used to calculate the rates in the experiments when the concentration of methylglyoxal was 2·16 mM. The calculated rates differ from the observed rates by an amount much greater than the experimental error. For example, the calculated rates (expressed as ΔE/min.) at concentrations of GSH of 0·162, 0·216 and 0·432 mM are 0·0182, 0·0229 and 0·0368 respectively, whereas the observed rates are 0·0230, 0·0285 and 0·0480. The conclusion is that the mechanism put forward by Kermack & Matheson does not accommodate the finding that the velocity is a symmetrical function of the concentrations of the substrates.

The most direct evidence that Kermack & Matheson (1957) put forward for their view that (free) glutathione was the substrate was based on an experiment in which the enzymic reaction appeared to proceed more slowly after an equilibrium concentration of the hemimercaptal had been attained. This experiment we have repeated, and our results (Fig. 6) show no such difference. The formation of hemimercaptal is quite rapid, and the attainment of equilibrium (as measured by the change in E) is characterized by a half-life of the order of 10 sec.

Petrovicki (1939) and Crook & Law (1952) found that the hemimercaptal from methylglyoxal and GSH was unreactive, as judged by the formation of lactic acid from *S*-lactoylglutathione. Excess of methylglyoxal (perhaps owing to the presence of impurities) apparently catalyses the hydrolysis of *S*-lactoylglutathione. The reported differences between the course of the overall reaction when the hemimercaptal is used (rather than a mixture of methylglyoxal and GSH) thus reside in the second (hydrolytic) step, and throw no light on the mechanism of the first step. We have, in fact, prepared the hemimercaptal by the method of Schubert (1935), and used it as the substrate: the rate of reaction was that expected on the assumption that the hemimercaptal rapidly forms the equilibrium mixture with methylglyoxal and GSH.

There are several mechanisms for an enzymic reaction between two substrates other than the one put forward by Kermack & Matheson (1957). These cannot be tested at all rigorously by the data at present available for the glyoxalase reaction; in all cases, the velocity would only be a symmetrical function of the concentrations of the substrates if several rate constants happened, fortuitously, to have values such that $\Phi_1 = \Phi_2$ in equation (4). Hence the simplest conclusion is that the hemimercaptal [$CH_3 \cdot CO \cdot CH(OH) \cdot SG$] is the substrate of glyoxalase.

This type of mechanism is not common in enzymic reactions. Another example may be provided by formaldehyde dehydrogenase; here Strittmatter & Ball (1955) have suggested that the substrate is *S*-hydroxymethylglutathione

[$CH_2(OH) \cdot SG$].

The reactions catalysed by glyoxalase I and by formaldehyde dehydrogenase are similar: both involve GSH and carbonyl groups, but that catalysed by glyoxalase is an intramolecular Cannizzaro reaction, whereas formaldehyde dehydrogenase requires diphosphopyridine nucleotide. Strittmatter & Ball also proposed a kinetic test for this type of mechanism. Consider two experiments, the first carried out at concentrations of methylglyoxal and GSH given by m_1 and g_1 respectively, and the second at concentrations

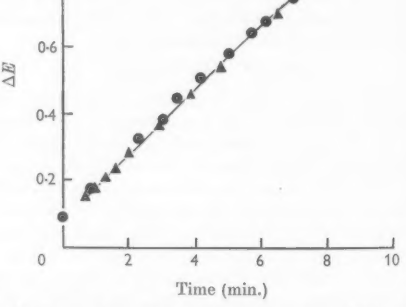


Fig. 6. Comparison of the rates of formation of *S*-lactoylglutathione from methylglyoxal and GSH, and from an equilibrium concentration of hemimercaptal. Both cells contained GSH (1 mM), and in one cell (●) methylglyoxal (2 mM) was present from the beginning, and in the other cell (▲) it was added after 70 min.; enzyme was then added immediately to both cells. The interval of time may be decreased to 5 min. without affecting the results.

given by m_2 and g_2. If the rate is the same in the two experiments, and if the rate depends on the concentration of hemimercaptal, then the concentration of hemimercaptal must be the same in the two experiments,

$$\text{i.e.} \quad (m_1-q)\,(g_1-q) = (m_2-q)\,(g_2-q) \qquad (7)$$

$$\text{or} \quad q = (m_2g_2-m_1g_1)/(m_2+g_2-m_1-g_1).$$

From smooth curves drawn through the experimental points (Fig. 1), pairs of values of the concentrations of the substrates at which the rates are equal were chosen. Values of q were then obtained from equation (7), and hence values of the equilibrium constant K were obtained. The values of K were in the range 1–2 mM, which is in reasonable agreement with the value (2 mM) obtained by the entirely independent method (the change in E on mixing the components) already described. The values for K, however, showed a trend towards higher values at lower concentrations of the substrates, which is not readily accounted for.

Rate of relatively high concentrations of glutathione

When the concentration of GSH is greater than that of methylglyoxal, further increase does not increase the rate of the enzymic reaction, but slightly decreases it (Kermack & Matheson, 1957). Thus, for a given concentration of methylglyoxal, there is an optimum concentration of GSH. This feature can be explained if the hemimercaptal (Q) is the substrate, and free glutathione (G) is an inhibitor. Then the mechanism (details of the formation of hemimercaptal are here omitted) would be:

$$\mathrm{M}+\mathrm{G} \rightleftarrows \mathrm{Q}$$

$$\mathrm{E}+\mathrm{Q} \rightleftarrows \mathrm{EQ}$$

$$\mathrm{E}+\mathrm{G} \rightleftarrows \mathrm{EG} \text{ equilibrium constant } K_G$$

$$\mathrm{EQ} \longrightarrow \mathrm{E}+\text{product}.$$

This mechanism leads to the equation:

$$\frac{1}{v_0} = \frac{1}{V_{\max.}} + \frac{K_m}{V_{\max.}}\left(1+\frac{g}{K_G}\right)\frac{1}{q} \qquad (8)$$

where g is the concentration of free GSH, and q is the concentration of hemimercaptal. If g_0 is the total concentration of GSH, then after the equilibrium concentration of the hemimercaptal has been built up

$$g = g_0-q.$$

If m_0 is the total concentration of methylglyoxal, the equilibrium constant for the formation of hemimercaptal is given by

$$K = (m_0-q)\,(g_0-q)/q.$$

It may be shown that the rate, given by equation (8), is greatest when the total concentration of GSH has the value

$$g_0 = [m_0+K+(KK_G)^{\frac{1}{2}}]/[1+(K/K_G)^{\frac{1}{2}}].$$

The calculation is not given, since the data are not adequate to test the equation. If the value of K_G were about 1 mM, however, a curve similar to that obtained by Kermack & Matheson would be obtained, and this value of K_G seems quite reasonable, as it is similar to the value of K_i obtained with ophthalmic acid. It seems probable that the suggestion that free GSH is an inhibitor in the glyoxalase reaction can account for the variation of the rate with the concentration of GSH. GSH is also an inhibitor in the reaction catalysed by glyoxalase II (Wieland, Pfleiderer & Lau, 1956).

SUMMARY

1. Ophthalmic acid, an analogue of glutathione that occurs in calf lens, was a competitive inhibitor in the reaction catalysed by glyoxalase I.

2. The velocity of the enzymic reaction is a symmetrical function of the concentrations of methylglyoxal and glutathione (GSH). This result suggests that the hemimercaptal,

$$\mathrm{CH_3{\cdot}CO{\cdot}CH(OH){\cdot}SG},$$

which is formed on mixing the components, is the substrate for glyoxalase I. Some supporting evidence for this suggestion has been provided, and a different mechanism, which had been proposed previously, has been excluded.

3. The initial rate of formation of the hemimercaptal is proportional to the concentration of methylglyoxal but independent of the concentration of glutathione. The rate-determining step in the formation of the hemimercaptal is probably the liberation of free methylglyoxal from a hydrate.

4. At low concentrations of methylglyoxal and high concentrations of enzyme the rate of the enzymic formation of *S*-lactoylglutathione is affected by the rate of the (non-enzymic) formation of hemimercaptal.

5. The retardation caused by a relatively high concentration of glutathione is ascribed to the action of free glutathione as an inhibitor (the hemimercaptal being the substrate).

We should like to thank our colleagues at this Laboratory for advice and encouragement, the National Council to Combat Blindness Inc., New York, for a Fight-for-Sight grant, and The Distillers Co. Ltd., for a gift of glutathione. One of us (E. E. C.) was in receipt of a Thornton Bursary.

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Studies in Detoxication

86. THE METABOLISM OF ^{14}C-LABELLED ETHYLENE GLYCOL*

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Ethylene glycol (ethane-1:2-diol) and its derivatives are regarded as relatively toxic substances to human beings, and the toxicology has been summarized by Browning (1953). This compound appears to be more toxic to man than to laboratory animals, for while oral LD_{50} doses vary from about 2 ml./kg. for cats to 13 ml./kg. for mice (Laug, Calvery, Morris & Woodard, 1939; Lehmann & Flury, 1943; Oettingen, 1943; Spector, 1956*a*), the minimum lethal dose for man is about 1·6 g./kg. Fatal poisoning from ethylene glycol in man is usually the result of the accidental drinking of anti-freeze fluid as a substitute for alcoholic beverages (e.g. Pons & Custer, 1946; Harger & Forney, 1959). The cause of death from ethylene glycol has been attributed to lesions of the central nervous system (Pons & Custer, 1946); renal damage due to deposition of calcium oxalate crystals in the renal tubules is usually not severe enough to cause death, although metabolic conversion into oxalate has often been postulated as the reason for the toxic effects. Most studies on the fate of the glycol in animals have been concerned with oxalate formation and excretion (see Oettingen 1943).

The only well established metabolite of ethylene glycol in man and animals is oxalic acid, which, however, accounts for less than 2% of the dose (see Oettingen, 1943). Glycolaldehyde, glyoxal, glycollic acid and glyoxylic acid are possible metabolites. Glycollic acid has been claimed to be a metabolite in rabbits (Mayer, 1903). Glucuronic acid conjugation of ethylene glycol has not been found (Fellows, Luduena & Hanzlik, 1947; Gessner, Parke & Williams, 1960). Unchanged ethylene glycol, however, is excreted by dogs (Nakazawa, 1950) and humans (Hjelt, Tamminen, Fortelius, Raekallio & Alha, 1958; Harger & Forney, 1959).

This paper shows that the major end product of ethylene glycol metabolism is respiratory carbon dioxide. The main metabolite excreted in urine is unchanged ethylene glycol; oxalic acid is a minor metabolite, the amounts of which depend upon the dose and the species of animal. Intermediates in the formation of carbon dioxide are glycolaldehyde and glyoxylic acid, and these were detected in liver-slice experiments.

* Part 85: Parke (1961).

MATERIALS AND METHODS

[$^{14}C_2$]Ethane-1:2-diol. [$^{14}C_2$]Ethylene (The Radiochemical Centre, Amersham, Bucks) was converted into ethanediol according to Milas & Sussman (1937). The [$^{14}C_2$]ethylene gas (26 mg., 0·5μc) was contained in an ampoule which was broken (with a piece of iron and a magnet) in a closed flask containing 1 ml. of a 5·8% (w/v) solution of hydrogen peroxide and 0·07 ml. of a 0·5% (w/v) solution of osmium tetroxide, both in anhydrous *tert.*-butyl alcohol. The flask was connected with an arrangement for adding, in the absence of air, non-radioactive ethylene. The reaction mixture was kept at room temperature for 72 hr. and then brought into contact with an excess of non-radioactive ethylene and kept until the appearance of black colloidal osmium oxide indicated total decomposition of the

hydrogen peroxide (48 hr.). Shaking of the reaction vessel was avoided since it tended to reduce the yield. Carrier ethanediol (1 g.) was added to the reaction mixture, which was then distilled in a Craig microdistillation apparatus, equivalent to eight theoretical plates at a reflux ratio of 1:5. The yield of ethanediol, b.p. 197°/760 mm., was 0·97 g. and the recovery of ^{14}C was 43% (223μc/g.). The purity of the radioactive glycol was determined by isotope dilution with pure ethanediol (b.p. 198°; n_D^{20}, 1·4310) and counted as the bisphenylurethane, m.p. and mixed m.p. 158° (see below). The purity was 98±2%.

Animals and dosing. Chinchilla rabbits, albino rats, guinea pigs and cats were used. The ethanediol dissolved in water was administered either by mouth or by subcutaneous injection.

Determination of radioactivity. The radioactivity of the urine, faeces, tissues and expired air were determined as described by Parke (1956).

Determination of metabolites

Ethane 1:2-diol. Carrier ethanediol (400 mg.) was added to the radioactive urine (10 ml.), which was then continuously extracted with ether for 12 hr. The extract was dried with anhydrous Na_2SO_4 and evaporated. The residue was treated with phenyl *iso*cyanate (0·8 g.) and the mixture heated under reflux on a water bath for 0·5 hr. Excess of phenyl *iso*cyanate was allowed to evaporate on the water bath. The resulting crude bisphenylurethane of ethanediol was extracted with light petroleum (b.p. 100–120°) to free it from any carbanilide, and then recrystallized to constant activity from ethanol. The bisphenylurethane had m.p. and mixed m.p. 158°.

Oxalic acid. This was determined according to Parke & Williams (1953).

Hippuric acid. To the urine (10 ml.) was added hippuric acid (500 mg.) in a little 2N-NaOH. The urine was acidified with 2N-H_2SO_4 or conc. HCl and the precipitated hippuric acid collected by centrifuging or by filtration. Dissolution in alkali and precipitation with acid were repeated twice. The hippuric acid (m.p. 187°) was recrystallized from water to constant activity and then converted into its *p*-bromophenacyl ester (m.p. and mixed m.p. 151°), which was recrystallized from ethanol.

Glycolaldehyde and glyoxal. Both these aldehydes give the same osazone. Glyoxal (260 mg. of a 30% solution) was added to the urine (10 ml.). This was immediately followed with one drop of saturated aq. Na_2SO_3 to prevent resin formation from the aldehyde. The urine was then treated with 0·5 ml. of a solution of phenylhydrazine (1 ml. of phenylhydrazine in 1 ml. of acetic acid diluted with 5 ml. of water) and the phenylosazone separated immediately. The osazone was recrystallized from ethanol until the activity disappeared (m.p. and mixed m.p. 179°).

Glycollic acid. The acid (600 mg.) was added to urine (10 ml.), followed by 2N-$CuSO_4$ (5 ml.), and the solution adjusted to pH 7 with 2N-NH_3 solution. After the solution had been kept at 0° for 6 hr., the copper glycollate was collected and suspended in water, and the copper removed with H_2S. The solution was then evaporated to dryness and the residue (110 mg.) was heated with aniline (0·5 g.) at 130°. The product, glycollic acid anilide (m.p. and mixed m.p. 97°), was recrystallized from water until the activity disappeared.

Glyoxylic acid. The acid (200 mg.) was added to urine (5 ml.) and the mixture treated with a solution of 2:4-dinitrophenylhydrazine (1 g. of 2:4-dinitrophenylhydrazine in 3 ml. of conc. H_2SO_4 made up to 30 ml. with aq. 80% ethanol) until no further precipitation occurred. The glyoxylic acid 2:4-dinitrophenylhydrazone was recrystallized from aq. ethanol until the activity disappeared (m.p. and mixed m.p. 190°; cf. Ratner, Nocito & Green, 1944).

Urea. Urea (1 g.) was dissolved in urine (about 20 ml.) and conc. HNO_3 (5 ml.) was added. The urea nitrate was filtered off and then dissolved in water (20 ml.). The solution was treated with charcoal and filtered, and 5 ml. of conc. HNO_3 was added to precipitate urea nitrate. This purification was repeated. The salt was then dissolved in 5 ml. of water, and the solution was neutralized with Na_2CO_3 and evaporated to dryness. The urea was extracted with ethanol and then the extract was evaporated. The residue of urea was treated with two equivalents of a 7% (w/v) solution of xanthydrol in acetic acid and the mixture refluxed for 0·5 hr. The bisxanthyl derivative of urea (m.p. and mixed m.p. 274°) was recrystallized from aqueous dioxan to constant activity.

Isotope-dilution analyses were also carried out for uric acid, carbonate, pyruvic acid, acetaldehyde, acetic acid and acetone in urine, but none of these compounds were found as metabolites of ethanediol.

Tissue-slice experiments. Rat-liver slices (2 g.) were prepared as described by Umbreit, Burris & Stauffer (1949) and Elliott (1955). The slices were incubated at 37° for 2 hr. in 25 ml. of buffered Krebs–Ringer phosphate solution, pH 7·4 (see Umbreit *et al.* 1949), which had been made 0·01M with respect to the substrates. Incubations were carried out with [$^{14}C_2$]ethanediol alone, with [$^{14}C_2$]ethanediol + glycolaldehyde and with [$^{14}C_2$]ethanediol + glyoxylic acid. At the end of 2 hr. the Ringer solution was filtered from the slices. The CO_2 in the solution was precipitated and counted as $BaCO_3$. Glycolaldehyde was estimated by adding 0·2 g. of carrier aldehyde and isolating it as the phenylosazone or the 2:4-dinitrophenylosazone (m.p. and mixed m.p. 310°, from acetone); since glyoxal forms the same osazones this procedure included any glyoxal present. Glyoxylic acid was estimated by adding 70–80 mg. of carrier, which was isolated as the 2:4-dinitrophenylhydrazone.

RESULTS

Oxidation to carbon dioxide. Table 1 shows the distribution of ^{14}C in the expired air and urine of rabbits receiving [$^{14}C_2$]ethanediol by injection or by mouth. In doses of 0·1–2·0 g./kg. some 20–30% of the radioactivity is excreted in the urine. With the smaller doses of 0·124 g./kg., a large proportion of the ^{14}C is eliminated in the expired air as carbon dioxide and in one animal the amount was 60% of the dose in 3 days (see Fig. 1). Table 1 suggests that with the smaller doses of ethanediol about a quarter is eliminated, mainly unchanged, in the urine, and the main bulk of the rest is oxidized to carbon dioxide. This may be true also for doses up to 1–2 g./kg. Above 2 g./kg. ethanediol is toxic and large amounts are excreted in the urine, presumably unchanged. Table 2 gives similar observations on

rats; it suggests that as the dose rises to about 1 g./kg. a greater proportion of the dose is eliminated in the urine and a smaller proportion is oxidized to carbon dioxide.

Metabolites in urine. Table 3 shows the results of isotope-dilution experiments on the urine of rabbits receiving small doses of [$^{14}C_2$]ethanediol. The main excretory product is the unchanged diol, which accounts for most of the radioactivity of the urine. Gessner *et al.* (1960) showed that ethanediol does not form a conjugated glucuronic acid. The only other labelled compound found in the urine in appreciable amounts (about 1 % of the dose) was urea. Traces (0·1 % or less) of oxalic acid were found, but not glycolaldehyde, glyoxal, glycollic acid or glyoxylic acid. No evidence was obtained for the excretion of any other radioactive metabolite.

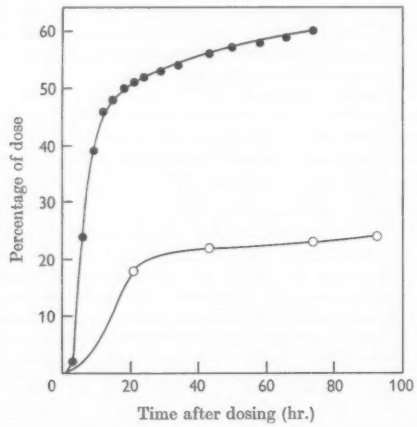


Fig. 1. Excretion of the total radioactivity in the urine (○) and $^{14}CO_2$ in expired air (●) by a rabbit which had received 0·124 g. of [$^{14}C_2$]ethanediol/kg. orally.

Formation of oxalic acid. Table 3 shows that with small doses of ethanediol very little is converted into and excreted as oxalate by the rabbit. The formation of oxalate was therefore investigated by the isotope-dilution technique with larger doses and with other species of animals. Table 4 shows that there are species differences in the amount in urine of oxalic acid formed from ethanediol. Cats form much more oxalic acid than do the other species examined and do not survive doses of 1 g. of ethanediol/kg. In rabbits and guinea pigs the oxalate excretion hardly reached 0·5 % of the dose at any level tried. Rats seem to be intermediate between cats and the other two species. Doses of 1 g. of ethanediol/kg. were not fatal to rats, rabbits and guinea pigs.

Table 1. *Elimination of* ^{14}C *by rabbits receiving* [$^{14}C_2$]*ethanediol*

Dose (g./kg.)	Time after dosing (days)	% of dose of ^{14}C in Urine	% of dose of ^{14}C in Expired air as CO_2
0·10*	2	21	—
0·124	1	21	42
0·124†	3	23	60
0·25*	2	22	—
0·25‡	11	17	—
0·25	11	21	—
0·50*	2	25	—
1·00*	2	26	—
1·50*	2	28	—
2·00	2	26	—
2·50*§	2	46	—
5·00‖	1	56	—

* These doses were given by subcutaneous injection; the others were oral.

† The animal receiving this dose eliminated 1 % of the dose in the faeces and its tissues contained 11 %. The total amount accounted for was thus 95 % of the dose.

‡ The animal receiving this dose eliminated 1 % of the ^{14}C in the faeces.

§ This dose produced symptoms of poisoning.

‖ The animal receiving this dose died 36 hr. later.

Table 2. *Fate of the* ^{14}C *in rats receiving* [$^{14}C_2$]*ethanediol by subcutaneous injection*

The dose of ^{14}C was 1–4 μc. The actual amount of ethanediol oxidized to respiratory CO_2 is given in parentheses.

Dose of glycol (g./kg.)	Wt. of rat (g.)	% of dose of ^{14}C after 24 hr. in Expired air as CO_2	Urine	Body tissues
0·06	115	—	21	18
0·10	160	—	21	13
0·10	300	23 (7 mg.)	35	—
0·60	250	19 (29 mg.)	55	—
1·00	280	14 (39 mg.)	58	—
5·00	320	6* (96 mg.)	32*	—
7·5	250	3·8* (71 mg.)	—†	—
10·0	225	2·4* (54 mg.)	—†	—

* In 12 hr. after dosing; the doses in these cases were fatal.

† No urine obtained.

Table 3. *Metabolites of ethanediol in rabbit urine*

[$^{14}C_2$]Ethanediol was given orally.

Expt. no.	1	2	3
Wt. of rabbit (kg.)	4·5	3·5	2·9
Dose of ethanediol (mg./kg.)	25	25	125
Dose of ^{14}C (μc)	17·1	14·1	80
Metabolites sought	Percentage of dose in 24 hr.		
Ethanediol	15·1	6·0	10·3
Oxalic acid	0·08	0·11	0·01
Urea	1·4	1·5	0·65
Hippuric acid	0	0	0
Total metabolites	16·6	7·6	11·0
^{14}C excreted in 24 hr.	18·2	9·5	18·0
(^{14}C excreted in 48 hr.	19·4	10·0	22·2)

Table 4. *Urinary excretion of labelled oxalic acid by animals dosed with* [$^{14}C_2$]*ethanediol*

The figures are the percentages of the dose of ^{14}C excreted in the urine in 48 hr.

	Cat		Rat		Rabbit		Guinea pig	
Dose (g./kg.)	Total ^{14}C in urine	^{14}C in oxalate	Total ^{14}C in urine	^{14}C in oxalate	Total ^{14}C in urine	^{14}C in oxalate	Total ^{14}C in urine	^{14}C in oxalate
0·10	78	0·72	21	0	21	0·07	26	0
0·25	59	3·65	41	0·97	22	0·05	24	0·06
0·50	43	2·00	45	0·57	25	0·15	27	0·01
1·00*	44†	0·87†	49	1·08	26	0·27	37	0·69
1·50	—	—	—	—	28	0·25	—	—
2·50‡	—	—	53	0·98	46	0·45	—	—
5·00‡	—	—	60†	0·48†	56†	0·37†	—	—
7·5‡	—	—	79†	0·83†	—	—	—	—

* This dose is fatal to cats within 48 hr. † 24 hr. urine. ‡ These doses were fatal in 24–48 hr.

Table 5. *Conversion of* [$^{14}C_2$]*ethanediol into labelled hippuric acid in the rat*

The rats were injected subcutaneously with [$^{14}C_2$]ethanediol (2 m-moles/kg.) and sodium benzoate (2 m-moles/kg.) simultaneously and the urine, collected for 24 hr., examined for hippuric acid by isotopic dilution.

Expt. no.	1	2	3
Wt. of rat (g.)	150	150	170
Dose of ethanediol (mg.)	15·3	15·7	22·0
Dose of ^{14}C (μc)	2·5	2·6	4·92
Dose of sodium benzoate (mg.)	43	43	50
Radioactivity of hippuric acid excreted in 24 hr. (% of dose)	9·6	8·4	7·0

Conversion of ethanediol into glycine. Weinhouse & Friedmann (1951) have shown that glyoxylic acid is converted into glycine, oxalate and carbon dioxide in the rat. Glycollic acid is also converted into glycine and carbon dioxide, but it is not readily converted into oxalate. If glyoxalate and glycollate are intermediates in the metabolism of ethanediol, then the simultaneous administration of benzoic acid with [$^{14}C_2$]ethanediol to rats should yield radioactive hippuric acid. Table 5 shows the results of three such experiments in which about 8% of the radioactivity of the administered ethanediol was incorporated into glycine.

Formation of glycolaldehyde and glyoxylic acid from ethanediol. Tables 1 and 2 suggest that a major portion of ethanediol is oxidized to carbon dioxide in the rabbit and rat. It is known that oxalic acid is not oxidized to carbon dioxide appreciably by animal tissues (Weinhouse & Friedmann, 1951; Curtin & King, 1955; Brubacher, Just, Bodur & Bernard, 1956). It is therefore unlikely that ethanediol is converted into carbon dioxide via oxalic acid. It is more probable that it is oxidized via intermediates such as glycolaldehyde, glycollic acid and glyoxylic acid. However, none of these intermediates were found in the urine. [$^{14}C_2$]Ethanediol was incubated with rat-liver slices alone and in the presence of glycolaldehyde and glyoxylic acid. The results given in Table 6 show that, when [$^{14}C_2$]ethanediol is

Table 6. *Conversion of ethanediol into carbon dioxide, glycolaldehyde and glyoxylic acid in rat-liver slices*

[$^{14}C_2$]Ethanediol (0·01 M) was incubated with 3 g. (fresh wt.) of rat-liver slices suspended in 25 ml. of Krebs–Ringer phosphate soln. for 2 hr. at 37° in an atmosphere of O_2. Glycolaldehyde and glyoxylic acid were added in final concn. of 0·01 M as trapping agents, and isotope dilutions for these substances were determined by procedures described in the text. The values given are in microatoms of labelled substrate carbon incorporated into the metabolite/g. of dry tissue/hr. (see Nakada, Friedmann & Weinhouse, 1955).

Substrates	Products: Carbon dioxide			Glycolaldehyde*			Glyoxylic acid		
Expt.	1	2	3	1	2	3	1	2	3
[$^{14}C_2$]Ethanediol alone	6·4,	8·7,	7·3	7·0,	10·8,	9·4	0·3,	0·4,	0·1
[$^{14}C_2$]Ethanediol + glycolaldehyde	1·1,	1·6,	1·2	3·7,	2·6,	4·4	—	—	—
[$^{14}C_2$]Ethanediol + glyoxylic acid	1·0,	1·4,	1·0	—	—	—	2·0,	0·9,	0·6

* Includes glyoxal.

incubated with liver slices, labelled carbon dioxide and labelled glycolaldehyde and/or glyoxal are formed. Labelled glyoxylic acid is just detectable. When [$^{14}C_2$]ethanediol is incubated in the presence of unlabelled glycolaldehyde or glyoxylic acid as trapping agent there is a marked reduction in the ^{14}C of the respiratory carbon dioxide with both substances and a significant increase in the labelling of glyoxylic acid. On the basis of the criteria suggested by Nakada, Friedmann & Weinhouse (1955), these experiments indicate that both glycolaldehyde and glyoxylic acid are on the pathway of oxidation of ethanediol to carbon dioxide in the rat-liver slice.

DISCUSSION

The present work shows that, in the rat and rabbit, two main excretory products of ethylene glycol are carbon dioxide eliminated in the expired air and unchanged ethylene glycol eliminated in the urine. Table 1 shows that 17–28 % of the ^{14}C administered to rabbits is excreted in the urine, when the dose of the glycol is 0·1–2·0 g./kg. The results in Table 3 suggest that most of the urinary radioactivity is due to unchanged ethylene glycol, for, apart from labelled urea and traces of oxalic acid, no other labelled compound could be identified in the urine. At low doses (0·12 g./kg.) most of the administered [$^{14}C_2$]ethanediol appeared as carbon dioxide. One rabbit which excreted 23 % of the dose of ^{14}C in the urine and 60 % in the expired air as carbon dioxide in 3 days (Table 1) also had 11 % of the ^{14}C left in its tissues and 1 % in the faeces, so that 95 % of the administered ^{14}C was accounted for. From Fig. 1 it can be seen that half the dose of ethylene glycol was oxidized to and eliminated as carbon dioxide in 18 hr. after dosing. Table 1 also shows that at doses of 2·5 and 5 g./kg. nearly one-half of the administered radioactivity is eliminated in the urine. This suggests that at the higher doses more of the glycol is being eliminated unchanged. Similar results were obtained for rats (Table 2). These findings support the view that the toxicity of ethylene glycol may be partly due to the unchanged glycol (cf. Pons & Custer, 1946).

It can be assumed (see Table 3) that the major compound in the urine is the unchanged glycol. In the guinea pig, little or no oxalate is excreted until the dose reaches 1 g./kg. (Table 4). The minimal fatal dose of the glycol by mouth for guinea pigs has been given as 7·4 g./kg. (Laug *et al.* 1939) and 6 g./kg. (Smyth, Seaton & Fischer, 1941). In the rabbit, the oxalate output is also small and even at 5 g./kg. the oxalate output in 24 hr. is no more than 0·37 % of the dose, although 56 % of the dose had been eliminated in the urine. The oral lethal dose of ethylene glycol for rabbits is about 9 g./kg. (Lehmann & Flury, 1943). The glycol is, therefore, not very toxic to rabbits and guinea pigs, and these species do not produce large amounts of urinary oxalate. This would suggest that, at least for rabbits, toxicity is partly due to the unchanged glycol. Cats seem to produce very much more oxalate than rabbits; further, cats eliminate a large proportion of the administered ^{14}C in the urine (see Table 4). We have found that 1 g./kg. of the glycol was fatal to cats but had no effect on rats, rabbits and guinea pigs. Spector (1956*a*) recorded that the subcutaneous lethal dose of ethylene glycol for cats is 2 g./kg. Thus in cats the toxicity of the glycol could be due to the unchanged glycol and to oxalate formation. Rats excrete appreciably more oxalate in urine than guinea pigs and rabbits and further they seem to excrete more of the administered ^{14}C in the urine. The oral minimum lethal dose of the glycol for rats has been given as 5·5 g./kg. (Laug *et al.* 1939) and 7·7 g./kg. (Smyth *et al.* 1941). Oettingen (1943) recorded that the glycol is consistently more toxic to rats than to rabbits. From a comparison of non-

lethal doses (see Table 4) it appears that ethylene glycol is more toxic to the species which excrete the most oxalate and the most unchanged glycol in the urine, and is least toxic to the species which are more able to oxidize it to carbon dioxide.

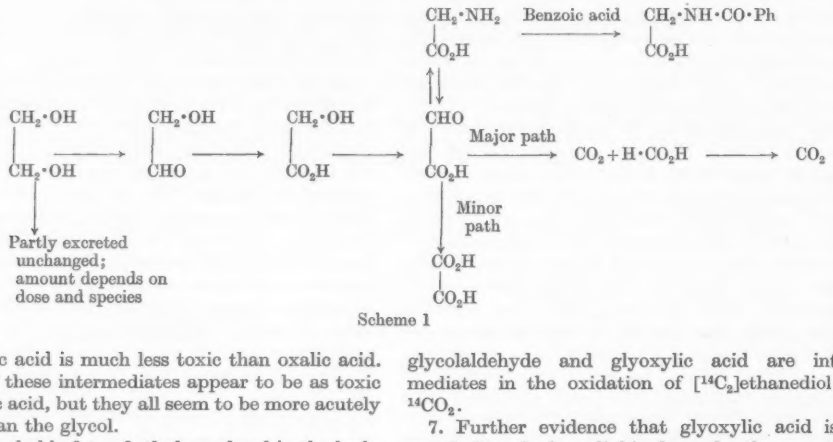
The distribution of ^{14}C in some rat tissues is given in Table 7. The brain was examined because Pons & Custer (1946) found some oxalate crystals in the brains of humans fatally poisoned by ethylene glycol; apart from the kidney no other tissues contained oxalate crystals. The concentration of ^{14}C in the brain of rats after 4 days was less than in most tissues (Table 7). The concentration of ^{14}C was much greater in liver, kidney and bone. It seems likely that the ^{14}C in bone is present as oxalate, for Curtin & King (1955) have shown that, in rats killed 10 days after an intraperitoneal injection of [^{14}C]oxalate, the bones contained more ^{14}C/g. than did any other tissue.

Oxidation of ethylene glycol to carbon dioxide. The metabolites of ethylene glycol have been shown to be oxalic acid and carbon dioxide in the whole animal (Tables 1–4) and glycolaldehyde and glyoxylic acid in the rat-liver slice (Table 6). Since oxalic acid is not readily oxidized to carbon dioxide *in vivo* (e.g. Weinhouse, 1955), then the glycol must be converted into carbon dioxide via an intermediate lying between it and oxalic acid. The major pathway for the oxidation of glycolaldehyde in animal tissues as suggested by the work of Weinhouse & Friedmann (1952), Nakada *et al.* (1955) and Friedmann, Levin & Weinhouse (1956) (see also Weinhouse, 1955) is: glycolaldehyde → glycollic acid → glyoxylic acid → carbon dioxide + formic acid → carbon dioxide. On this basis glyoxylic acid would be the precursor of carbon dioxide. Glyoxylic acid is known to be converted into carbon dioxide, glycine and oxalate in the whole rat and in liver preparations (Weinhouse, 1955). This work shows that ethylene glycol is also converted into carbon dioxide (Table 1), glycine (Table 5) and oxalate (Table 4). Glyoxylic acid is therefore the probable precursor of both the carbon dioxide and oxalate formed from the glycol. However, Weinhouse (1955) showed that the extent to which glyoxylic acid is converted into oxalate in rat-liver homogenates depends on its concentration, for at relatively low concentrations glyoxylate is oxidized entirely to carbon dioxide, but at relatively high concentrations it is partly oxidized to oxalate. The increase in oxalate excretion with increasing dose of ethylene glycol observed in the rabbit (see Table 4) could therefore be explained by the formation of increasing concentrations of glyoxylate in the tissues. The difference in oxalate excretion between cats and rabbits (see Table 4) is more difficult to explain, for cats also excrete considerably more of the radioactivity in the urine than do rabbits. This suggests that cats do not oxidize the glycol to the various intermediates given above as efficiently as rabbits, but at the same time cats appear to oxidize glyoxylic acid to oxalic acid more readily than rabbits. Glycolaldehyde, glycollic acid and glyoxylic acid were not found in the urine of rabbits dosed with ethylene glycol but, since they are probable intermediates in the oxidation of the glycol, their participation in the toxic effects should be considered. According to Pohl (1923*a*), 4 g. of oxalic acid by mouth (i.e. about 60 mg./kg.) can be fatal to man and about 100 mg./kg. injected subcutaneously can be fatal to rabbits and cats. The subcutaneous lethal dose of glycolaldehyde for rabbits is 4 g./kg. (Mayer, 1903; Spector, 1956*b*). The intravenous lethal dose of glycollic acid for cats has been given as 1 g./kg. (Merck Index, 1960): according to Riker & Gold (1942) 0·1 g. of sodium glycollate/kg. rarely causes toxic effects in cats and dogs, 0·25 g./kg. is toxic but rarely fatal, whereas 0·5 g./kg. may prove fatal. In herbivores, chronic daily feeding of 300 mg. of sodium glycollate/kg. is tolerated without damage and higher doses up to 1 g./kg. can be tolerated for short periods (Scholz, 1956). According to Pohl (1923*b*)

Table 7. *Distribution of* ^{14}C *in certain tissues of rats 4 days after dosing with* [$^{14}C_2$]*ethanediol*

Animal	Dose of glycol (g./kg.)	% of dose of ^{14}C in				Sp. activity/g. of tissue (relative to liver = 1)			
		Liver	Kidney	Brain	Bone	Liver	Kidney	Brain	Bone
Rabbit	0·10	1·4	0·2	0·01	1·7	1	0·7	0·1	0·3
Rat	0·06	2·2	0·5	0·04	8·2	1	0·9	0·1	1·2
	0·10	2·8	0·3	0·03	5·3	1	0·4	0·04	0·7
	1·00	1·3	0·25	0·11	9·6	1	0·5	0·4	2·0
	2·50	0·8	0·2	—	4·2	1	0·8	—	1·5
	5·00	0·5	0·1	0·02	3·7	1	1·1	0·3	0·3
	7·5	0·4	0·2	0·02	2·9	1	1·3	0·2	1·7
	10·0*	1·3	1·4	0·10	4·0	1	1·8	0·3	0·7
					Mean for rats		0·97	0·22	1·16

* The animal receiving this dose died on the fourth day.



Scheme 1

glyoxylic acid is much less toxic than oxalic acid. None of these intermediates appear to be as toxic as oxalic acid, but they all seem to be more acutely toxic than the glycol.

The probable fate of ethylene glycol in the body is shown in Scheme 1.

SUMMARY

1. [$^{14}C_2$]Ethane-1:2-diol has been prepared from [$^{14}C_2$]ethylene and its fate in the animal body investigated.

2. In the rabbit, at dose levels of 0·1–2·0 g./kg. about 20% of the dose of radioactivity is excreted in the urine in 2 days, mainly as unchanged ethanediol, with smaller amounts as urea and oxalic acid; at higher doses (2·5 and 5·0 g./kg.), the radioactivity in the urine increases to about 50%. At a dose level of 0·1 g./kg. about 40% of the glycol is eliminated as $^{14}CO_2$ in the expired air in 1 day, and 60% in 3 days. The remainder of the activity is mostly located in the tissues.

3. In the rat, the radioactivity excreted in the urine in 24 hr. increases from 21% at a dose level of 0·1 g./kg. to 58% at 1·0 g./kg., and the $^{14}CO_2$ in the expired air decreases from 23% in 24 hr. at 0·1 g./kg. to 2·4% in 12 hr. at 10 g./kg.

4. Rabbit and guinea pig excrete only traces (about 0·05% of dose) of oxalate in the urine at dose levels of ethanediol of less than 1 g./kg., which increases (about 0·5% of dose) at doses of 1–5 g./kg. Larger amounts of oxalate are excreted by rats (0·5–1·1% of dose) and by cats (0·7–3·7% of dose). At dose levels of 0·1–1 g. of [$^{14}C_2$]ethanediol/kg., the total radioactivity excreted in the urine by cats (43–78%) is greater than that excreted by rats (21–49%), rabbits (21–26%) or guinea pigs (26–37%), but both rats and rabbits show considerable increases at higher doses.

5. The concentration of radioactivity in the tissue of rats was greatest in liver, kidney and bone.

6. It has been shown in rat-liver slices that glycolaldehyde and glyoxylic acid are intermediates in the oxidation of [$^{14}C_2$]ethanediol to $^{14}CO_2$.

7. Further evidence that glyoxylic acid is a metabolite of ethanediol is shown by the excretion of [^{14}C]hippuric acid by rats injected with [$^{14}C_2$]-ethanediol and benzoic acid simultaneously.

8. The relative toxicity of ethanediol in different species, probable metabolic pathways and the toxicities of the metabolic intermediates are discussed.

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Induction and Repression of β-Galactosidase in Non-growing *Escherichia coli*

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It has been known since the earliest studies of enzyme induction that glucose will often prevent the induced synthesis of enzymes concerned with the metabolism of other carbohydrates. The glucose effect and similar phenomena have been summarized in a number of reviews (Monod & Cohn, 1952; Mandelstam, 1956; Pollock, 1959). It appears to be an instance of a more general type of reaction, now termed repression (Vogel, 1957), in which the rate of synthesis of an enzyme is specifically decreased by a repressor, which is generally, but not necessarily, a normal metabolite of the cells.

The inhibitory effect of glucose upon the induced synthesis of β-galactosidase in growing cultures of *Escherichia coli* has been examined by Cohn & Horibata (1959). The present experiments show that, in non-growing cells (i.e. in the absence of net protein synthesis), the repressor action of glucose is not specific, and that the synthesis of β-galactosidase is effectively inhibited by all substances tested which the cells can utilize as sources of carbon and energy.

MATERIALS AND METHODS

Organisms. The following strains of *E. coli* were used: ML 30 and K_{12} (wild type); ML 328c (Lac^+) (requiring leucine and able to form β-galactosidase); 160-37 (requiring arginine); 26-26 (requiring lysine); N.C.T.C. 8009 (requiring methionine); 15_T^- (requiring thymine); W 3301 (requiring uracil and biotin). All experiments were done at 35°. The wild-type strains were grown with shaking in a synthetic medium (pH 7·2) containing phosphate and inorganic salts (Mandelstam, 1958*a*) and glucose or another carbon source (1%, w/v). All organic acids were neutralized to pH 7·0 with NaOH; concentrations given in the text refer to the acid and not to its salt. For the auxotrophic mutants, the medium was supplemented with the appropriate growth factor as follows: DL-leucine (300 μg./ml.), L-arginine–HCl (100 μg./ml.), L-lysine–HCl (150 μg./ml.), L-methionine (100 μg./ml.), thymine (10 μg./ml.), uracil (100 μg./ml.), biotin (0·05 μg./ml.).

Induction of β-galactosidase. The cells were harvested when the culture contained about 0·8 mg. dry wt. of bacteria/ml., and were washed with phosphate buffer (having the same composition and pH as the phosphate in the growth medium). In the earlier experiments the bacteria were first starved of their specific requirement or, with the wild-type strains, of ammonium salts (see below). In later experiments this was omitted, and the cells, after washing, were suspended in the same phosphate buffer (at 0·2–0·4 mg. dry wt./ml.) with methyl β-D-thiogalactoside (MTG; 0·1 mg./ml.) as inducer. MTG was synthesized by the method of Helferich & Türk (1956).

Incorporation of [^{14}C]*leucine.* Washed bacteria were incubated in buffer as already described, with the addition of DL-[^{14}C]leucine (200 μg./ml.; specific activity about 30

counts/min./μg.). Samples (40 ml.) were taken at intervals. The precipitate obtained with trichloroacetic acid was prepared for counting at infinite thickness as described by Mandelstam (1958*b*). Carbon sources, when present, were used at 1% (w/v) unless otherwise stated. The suspensions were shaken in a water bath and samples (5 ml.) taken periodically and centrifuged immediately. This was found to stop the formation of β-galactosidase as effectively as the addition of chloramphenicol. The cells were treated with toluene in sodium phosphate buffer and β-galactosidase activity was measured as described by Rickenberg & Lester (1955), with *o*-nitrophenyl β-D-galactoside as substrate. A unit is defined as the amount of enzyme which liberates 1 μmole of *o*-nitrophenol/hr.

RESULTS

Effect of amino acid- or nitrogen-starvation upon the synthesis of β-galactosidase. Experiments were first carried out to determine to what extent cells starved of nitrogen or of an amino acid were capable of producing β-galactosidase.

Arginine-requiring cells were grown to 0·70 mg./ml. in glucose–synthetic medium, and divided into two equal portions. One was left in the flask at room temperature. The other was centrifuged, washed once with phosphate buffer (50 ml.), suspended in the same volume of medium with arginine omitted and shaken for 30 min. in a water bath at 35°. This period of starvation was previously shown to be sufficient to exhaust arginine and to cause the cells to accumulate and discharge into the medium considerable amounts of amino acids (Mandelstam, 1958*a*). Both the starved and unstarved bacteria were then centrifuged, washed once and suspended in 100 ml. of buffer containing MTG. In addition, the suspension of unstarved cells was supplemented with L-arginine–HCl (100 μg./ml.).

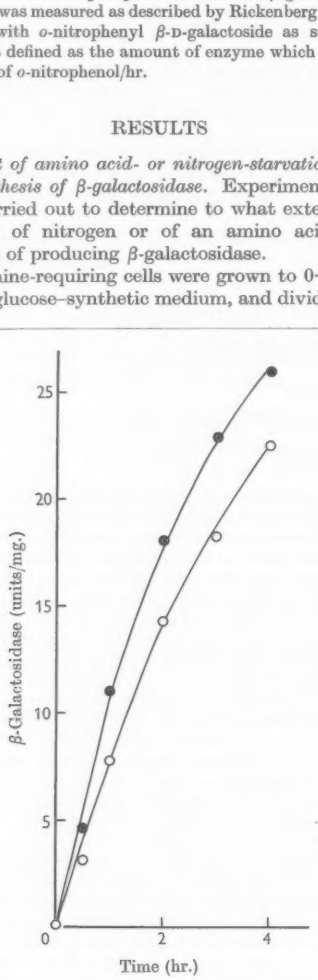

Fig. 1. Effect of arginine-starvation upon the subsequent production of β-galactosidase in washed suspensions. Arginine-requiring cells were starved for 30 min. (see the Materials and Methods section), washed and shaken in buffer with MTG. ○, Starved cells; ●, unstarved cells supplemented with L-arginine–HCl (100 μg./ml.).

Production of β-galactosidase in the shaken cultures is shown in Fig. 1. The starved cells formed the enzyme nearly as rapidly as the unstarved cells, the specific activities being 22·5 and 26 units/mg. dry wt. of bacteria at the end of 4 hr. It may be noted for comparison that cultures grown with lactose as carbon source contained about 100 units/mg. whereas cultures grown with succinate and MTG, which is a more efficient inducer, contained about 400 units/mg.

Similar experiments were carried out with wild-type *E. coli* (K_{12} and ML 30) and these were subjected to starvation for 30 min. by omitting ammonium salts from the medium. Again there was little or no impairment of the ability to produce β-galactosidase subsequently in washed suspensions, and this was also the case with cells which had been starved of leucine (see below), methionine or lysine.

Effect of thymine- or uracil-starvation upon the synthesis of β-galactosidase. The effect of uracil-starvation upon the ability of *E. coli* to form induced enzymes has been the subject of conflicting reports (see Pollock, 1959), and in thymine deficiency it has been reported (Cohen & Barner, 1954, 1955) that xylose-isomerase formation is not impaired. The effect of depriving *E. coli* of these pyrimidines has been examined.

Thymine-less *E. coli* (15_T-) was washed and suspended in glucose–synthetic medium (200 ml.) at 0·36 mg. dry wt./ml. and the suspension divided into two 100 ml. lots. Thymine (final concentration 10 μg./ml.) was added to one culture, and both were shaken in a water bath at 35°. For the first 60 min. growth in the two flasks was equal, but after this there was a progressive fall in the growth rate of the thymine-starved cells and growth had ceased after 100 min., when the bacterial density was 0·97 mg./ml. The unstarved culture had reached 1·3 mg./ml. at this stage. Portions of both cultures were centrifuged, washed and suspended in buffer (50 ml.) at the same bacterial density (0·35 mg./ml.). Thymine (final concentration 10 μg./

ml.) was added to the unstarved cells and MTG to both.

With the uracil-requiring strain (W 3301), washing was sufficient to deplete the cells and no growth occurred after resuspending the cells in glucose medium without uracil. Starvation was therefore continued for only 30 min., during which the culture containing uracil increased from 0·34 to 0·50 mg./ml. while the starved culture remained at 0·35 mg./ml. Both cultures were washed and resuspended at the same density (0·3 mg./ml.) in buffer (50 ml.) containing MTG. The unstarved cells were also given uracil (20 μg./ml.).

The results with uracil (see Fig. 2) are consistent with those of Pardee (1955), and show that uracil deprivation, under these conditions, does not significantly affect the ability to form β-galactosidase. The effect of longer periods of starvation was not investigated because it was found that starvation for 60 min. seriously damaged the cells. Oxygen uptake with glucose as substrate was decreased by over 50% (see also Cohen & Barner, 1955), and the cells failed to grow when subsequently transferred to a complete medium.

Thymine deficiency reduced the rate of formation of β-galactosidase by about 30% (Fig. 2).

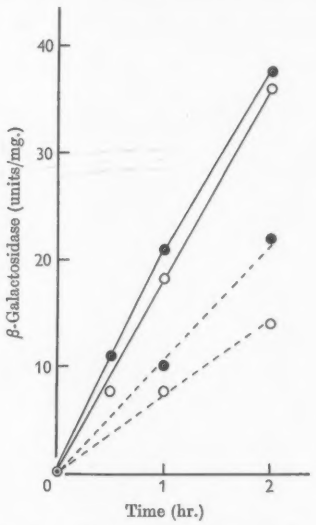

Fig. 2. Effect of pyrimidine-starvation upon the subsequent production of β-galactosidase in washed suspensions. Bacteria requiring thymine or uracil were starved (see the Materials and Methods section), washed and shaken in buffer with MTG. ○, Starved cells; ●, unstarved cells supplemented with pyrimidine (see text); —, uracil-requiring; - - - -, thymine-requiring.

Effect of a carbon source upon the synthesis of β-galactosidase. Leucine-requiring cells [ML 328c (Lac^+)] grown in succinate medium were washed and resuspended at 0·1 mg. dry wt./ml. in buffer containing MTG and Fe^{2+} and Mg^{2+} ions and the following additions: (*a*) none; (*b*) NH_4^+ ions; (*c*) succinate; (*d*) NH_4^+ ions and succinate; (*e*) NH_4^+ ions, succinate and leucine (i.e. complete medium). All additions were as in the growth medium. The cultures were shaken and samples taken for measurement of β-galactosidase and extinction.

The cells in complete medium grew almost without lag and the bacterial mass increased almost fourfold (0·1–0·38 mg./ml.) in 2 hr. This should be borne in mind when comparing enzyme production in this culture (see Fig. 3) with that of the four suspensions in which no growth occurred. The graph makes it apparent that omission from the complete medium of leucine alone or of leucine and ammonium salts led to complete repression of β-galactosidase. The further omission of succinate restored the ability to produce the enzyme although the rate was, of course, greatly below that observed in the growing culture.

The finding, that a carbon source which allowed a high rate of enzyme synthesis under normal growth conditions became totally inhibitory as soon as

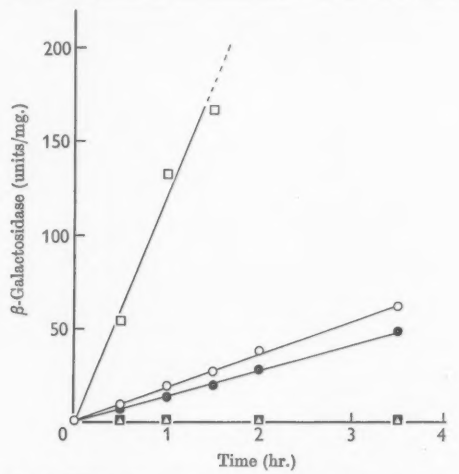

Fig. 3. Effect of succinate, ammonium salts and leucine upon production of β-galactosidase in leucine-requiring cells. Washed bacteria were shaken in buffer with MTG, Mg^{2+} ions, Fe^{2+} ions and further additions (see text) as follows: ○, none; ●, NH_4^+ ions; △, succinate; ■, NH_4^+ ions and succinate; □, NH_4^+ ions, succinate and leucine. The last-named is a complete medium, and the cells increased fourfold in 2 hr.; there was no growth in the other suspensions.

growth was prevented, appeared to hold generally for all the strains examined, and for all carbon sources tested (succinate, pyruvate, lactate, gluconate, glycerol, malate, fructose, maltose). The specific cause for the stoppage of growth was immaterial; that is omission of ammonium salts or of a specific amino acid or of a pyrimidine produced total inhibition of β-galactosidase synthesis, which was relieved by removal of the carbon source.

Comparison of β-galactosidase synthesis and the incorporation of DL-[^{14}C]*leucine into protein in the presence or the absence of succinate.* The possibility had to be examined that the inhibition of β-galactosidase synthesis was non-specific, i.e. in non-growing cells the presence of a carbon source might, for some reason, decrease protein synthesis generally.

For these experiments leucine-requiring cells grown in succinate–synthetic medium were washed and suspended (at 0·5 mg. dry wt./ml.) in buffer (200 ml.) containing MTG and DL-[^{14}C]leucine. A similar flask contained succinate (1 %, w/v) in addition. The suspensions were shaken at 35°. Samples (40 ml.) were taken every 40 min. and added to 1 ml. of chloramphenicol solution (1 mg./ml.) to stop further incorporation and enzyme synthesis. A sample (2·0 ml.) was taken for estimation of β-galactosidase and the rest of the suspension used to prepare protein for radioactivity measurement.

From Fig. 4 it is apparent that the formation of β-galactosidase bore little relation to the protein synthesis as measured by incorporation of leucine. The effect of succinate was to increase incorporation while decreasing the synthesis of β-galactosidase. The uptake of radioactivity into protein was similar to that found previously in several strains of *E. coli* and indicated a rate of protein turnover of about 5 %/hr. (see Mandelstam, 1958*b*).

Reversal of repression by malonate or 2:4-*dinitrophenol.* These results suggested that stopping growth caused the accumulation of metabolites which acted as repressors of β-galactosidase. It therefore seemed likely that suitable inhibitors might reverse the repression caused by carbon sources and that this might help to indicate what sort of metabolites the active repressor was derived from. Most of the experiments were carried out with W 3301 (requiring uracil), mainly because

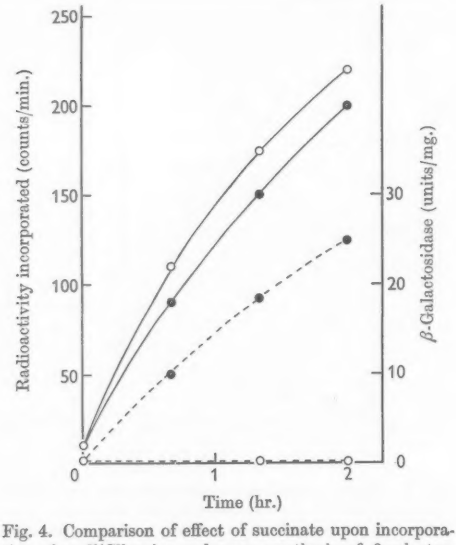

Fig. 4. Comparison of effect of succinate upon incorporation of DL-[^{14}C]leucine and upon synthesis of β-galactosidase. Leucine-requiring cells were washed and incubated in buffer with MTG and DL-[^{14}C]leucine. ○, With succinate; ●, no succinate. - - - -, β-Galactosidase; —, radioactivity incorporated into material insoluble in hot trichloroacetic acid (see the Materials and Methods section).

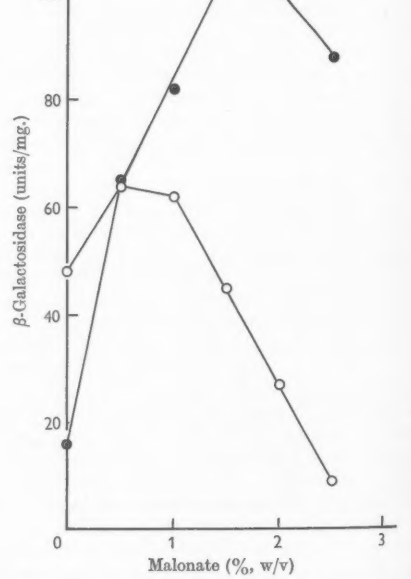

Fig. 5. Repression of β-galactosidase by succinate and reversal of repression by malonate. Washed cells were incubated in buffer with MTG and various concentrations of malonate, and β-galactosidase formation was measured after 2 hr. ○, No succinate; ●, with succinate (0·25% w/v).

this strain produced about twice as much enzyme in washed suspensions as most other strains of *E. coli*. However, some of the experiments have been repeated with ML 30 (wild-type) and M 328c (Lac^+) and similar results have been obtained.

(i) Malonate. It is well known that malonate competes with succinate for succinic dehydrogenase (Quastel & Wooldridge, 1928). Its effect upon the repression produced by succinate was examined as follows. Cells grown in glucose medium were washed and incubated in buffer (10 ml.) with MTG and various concentrations of malonate in the presence or the absence of succinate (0·25 %, w/v). The β-galactosidase formed after 2 hr. was measured. In the absence of succinate, malonate enhanced enzyme production (48–64 units) at a concentration of 0·5 % (w/v) whereas higher concentrations were inhibitory (Fig. 5). In the presence of succinate, and with no malonate, enzyme production was repressed (16 units). Malonate not only overcame the repression but at a concentration of 1·5 % allowed the production of about twice as much enzyme (106 units) as was found in the control suspension without either succinate or malonate (Fig. 5). Concentrations above 1·5 % produced some inhibition.

When these experiments were done with cells originally grown in succinate medium instead of glucose, the repression was not reversed by malonate at any concentration up to 2·5 %.

(ii) 2:4-Dinitrophenol. The bacteria were grown in synthetic medium with the compound to be tested as the sole source of carbon and energy. After washing, the cells were suspended as before in phosphate buffer, and various dilutions of 2:4-dinitrophenol in the same buffer were added. The suspensions (total volume 10 ml.) were induced with MTG as usual by shaking at 35° for 2 hr. and β-galactosidase was then measured. A similar set of flasks contained the carbon compound (final concentration 1 %, w/v).

Fig. 6 (*a*) shows the enzyme produced in 2 hr. as a function of 2:4-dinitrophenol concentration with and without succinate. In the absence of succinate there was a continuous fall in the rate of enzyme synthesis with increasing amounts of 2:4-dinitrophenol. In the presence of succinate, the repression (about 85 %) produced by succinate in the absence of 2:4-dinitrophenol was largely overcome at 0·125 mM-2:4-dinitrophenol. In these experiments the extent of the repression was somewhat variable but was never less than 80 %, and more often almost complete. Both the extent of reversal achieved and the concentration of 2:4-dinitrophenol producing the greatest effect also varied from one experiment to another. Thus the amount of enzyme synthesized in the presence of the optimum concentration of 2:4-dinitrophenol lay between 80 and 150 % of that produced in the control suspension without succinate (see Table 1), and the optimum concentration of 2:4-dinitrophenol varied from 0·125 to 0·25 mM.

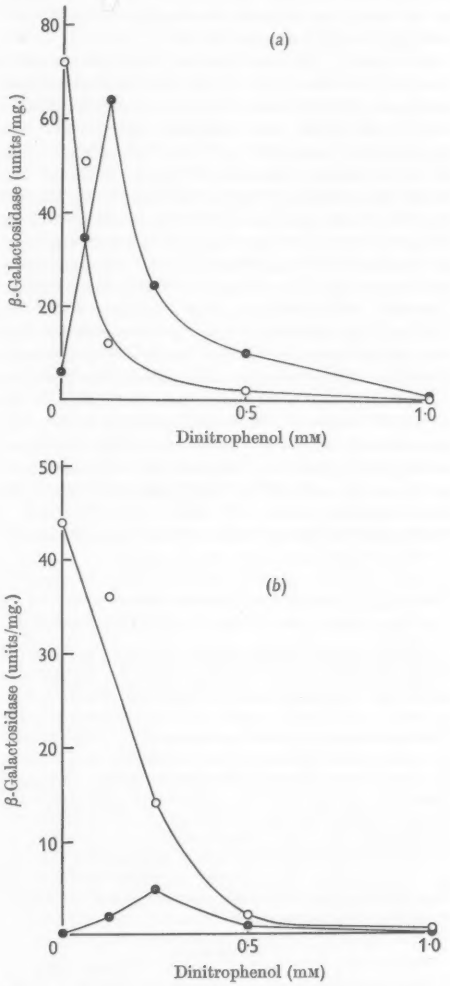

Fig. 6. (*a*) Repression of β-galactosidase by succinate and reversal of repression by 2:4-dinitrophenol. Washed cells were incubated in buffer with MTG and various concentrations of 2:4-dinitrophenol, and β-galactosidase formation was measured after 2 hr. ○, No succinate; ●, with succinate (1 %, w/v). (*b*) Repression of β-galactosidase by gluconate and partial reversal of repression by 2:4-dinitrophenol. Details were as given for (*a*). ○, No gluconate; ●, with gluconate (1 %, w/v).

The repression by succinate and the reversal with 2:4-dinitrophenol also depended upon the previous history of the cells. If the cells had originally been grown in glucose medium, the inhibition subsequently produced in washed suspension by succinate was less (about 50 % instead of 85–100 %) and it was reversed at a lower concentration of 2:4-dinitrophenol.

A number of other carbon compounds were tested in the same way. They could be divided into three groups. (*a*) Compounds producing repression which was often not complete and which was appreciably reversed by 2:4-dinitrophenol, i.e. 2:4-dinitrophenol restored 50 % or more of the ability to synthesize β-galactosidase. Succinate was typical of this group. (*b*) Compounds producing complete repression and exhibiting a low degree of reversal with 2:4-dinitrophenol, i.e. less than 10 % restoration of the ability to form the enzyme. Gluconic acid acted in this way (see Fig. 6*b*). (*c*) Compounds producing complete repression and not significantly reversible by 2:4-dinitrophenol. Glycerol and glucose are examples of this group.

The compounds tested are listed in Table 1. As might be expected, those compounds which are metabolized aerobically showed a high degree of reversibility with 2:4-dinitrophenol whereas those which can be utilized by anaerobic pathways were not reversible.

Repressor action of lactose. It has been shown by Rickenberg, Cohen, Buttin & Monod (1956) that galactosides are concentrated in *E. coli* by an active transport system which they called galactoside permease. The permease is induced in parallel with β-galactosidase and requires energy for its functioning (Kepes, 1960). The possibility had to be considered that the repressors produced their effect not by acting upon the enzyme-forming system but by preventing the entry of the inducer into the cells.

In an attempt to rule this out, the following experiment was done with lactose, which as a galactoside is transported into the cells by galactoside permease, and which as a carbon source should produce a repressor effect. Cells, grown in succinate medium, were induced in washed suspension in the usual way. At the end of an hour, when an appreciable amount of enzyme had been formed, the suspension was divided into three portions and the following additions were made: (*a*) none; (*b*) succinate (final concentration 1 %, w/v); (*c*) lactose (final concentration 1 %, w/v). Enzyme production was followed for the next 2 hr. The results (see Fig. 7) showed that in partially induced cells lactose could act as an efficient repressor in much the same way as succinate. The fact that it had this effect indicates that it was entering the cell and that the galactoside permease was therefore functioning.

Table 1. *Repression of β-galactosidase synthesis by carbon sources and the effect of 2:4-dinitrophenol*

Washed cells (W 3301) were incubated for 2 hr. with the compound (1 %, w/v) to be tested and MTG (0·1 mg./ml.) as inducer. 2·4-Dinitrophenol was added to give the following final concentrations (mM); 0, 0·125, 0·25, 0·5 and 1·0. The second column gives the concentration of 2:4-dinitrophenol at which the highest enzyme activity was obtained. The last column expresses the highest enzyme value in the series as a percentage of the control value obtained in the absence both of carbon source and of 2:4-dinitrophenol. The control value varied somewhat from one experiment to another but was in the range 40–60 enzyme units/mg. dry wt. of bacteria. 'Irreversible' indicates that enzyme formation was less than 2 % of the control at all concentrations of 2:4-dinitrophenol (DNP).

Compound	Optimum concn. of DNP (mM)	Reversibility (%)
Succinate	0·125–0·25	80–150
Lactate	0·5	50–80
Pyruvate	0·5	90–100
Malate	0·25	90–150
Gluconate	0·25–0·5	5–10
Glycerol	—	Irreversible
Glucose	—	Irreversible
Fructose	—	Irreversible
Maltose	—	Irreversible

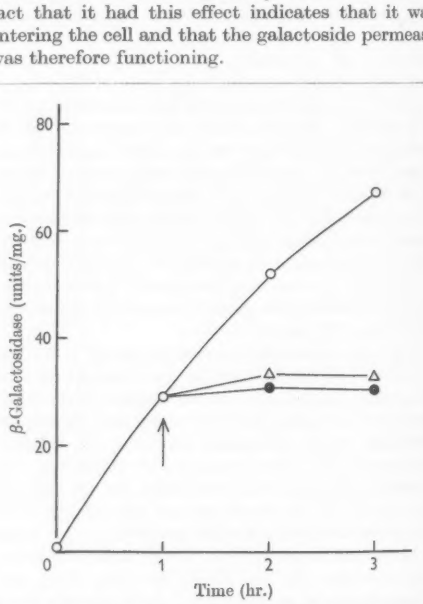

Fig. 7. Repression of β-galactosidase by succinate and by lactose. Washed cells were incubated in buffer with MTG. After 1 hr. the suspension was divided into three and additions were made as follows: ○, none; ●, succinate (1 %); △, lactose (1 %).

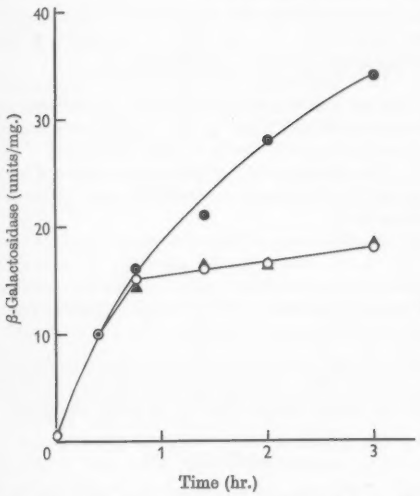

Fig. 8. Induction of β-galactosidase by lactose and MTG. Washed cells were incubated with phosphate buffer with additions as follows: ○, lactose; ●, MTG; ▲, MTG and lactose (1%).

If, however, lactose was added at the beginning of the incubation period together with MTG, the repressor effect was observed only after some enzyme had been formed, so that the lactose could be metabolized.

In Fig. 8 a comparison has been made of the induction of β-galactosidase by lactose and by MTG separately and together. It can be seen that lactose first acts as an inducer which is about as efficient as MTG, and then when sufficient enzyme has been formed, the repressor effect becomes manifest not only in the cells treated with lactose but in those treated with both MTG and lactose. These results make it reasonable to conclude that the repressor action of lactose is an effect upon the enzyme-forming system and imply that other carbon sources also act in this way and not upon the galactoside permease.

DISCUSSION

It was shown by Monod, Pappenheimer & Cohen-Bazire (1952) that in a growing culture of *E. coli* the amount of β-galactosidase formed was proportional to the net protein synthesis, i.e. the enzyme was a constant fraction of the total new protein being synthesized by the cells. In the present experiments, when the cells were starved of nitrogen or of an amino acid, there was no net synthesis of protein but there was nevertheless considerable production of β-galactosidase. The amount of enzyme formed in 1 hr. in the non-growing condition is about 10–30 units/mg. dry wt., depending upon the strain of *E. coli*. This rate of enzyme production should be considered in relation to the actual protein synthesis. It has been shown (Mandelstam, 1957, 1958*b*) that in non-growing *E. coli* protein is degraded and resynthesized at about 5%/hr. Thus for every 1% of protein 'turned over' in the presence of MTG, the bacteria acquire a specific enzyme activity of 2–6 units/mg. If this relationship could be maintained constant until all the protein had been degraded and resynthesized the activity of the cells would be 200–600 units/mg. This is the same order of activity as that of cells induced under conditions of normal growth. For instance, after growth for ten generations in lactose the activity is about 100 units/mg. and after growth in the presence of succinate and MTG it is about 400 units/mg. It is therefore apparent that β-galactosidase forms a more or less constant fraction of the total protein synthesized whether the cells are growing or not, provided, of course, that, in the latter condition, no carbon source is present. The kinetics of formation of β-galactosidase and its relationship to turnover during the period when cells are adapting to growth on a new carbon source have been discussed by Mandelstam (1960).

The inhibitory effect of a carbon source on the synthesis of β-galactosidase in a pyrimidine-less strain of *E. coli* had been noted by Pardee (1955). The present results show that the effect is general and that a carbon source becomes inhibitory as soon as growth is prevented by omitting any essential ingredient from the medium. This explains why Monod *et al.* (1952) obtained no β-galactosidase in *E. coli* auxotrophs deprived of their specific requirements.

Although β-galactosidase formation is inhibited by a carbon source, the synthesis of protein as measured by the incorporation of [^{14}C]leucine is, if anything, slightly enhanced. It follows that it may be misleading to use formation of β-galactosidase as an indication of the general ability of the cells to synthesize protein.

The results also show that the 'glucose effect' is not specific to glucose but can be produced by a variety of carbon sources, provided that growth of the cells is prevented. The fact that the repressor effect of these substances is manifest only under such conditions suggests that when growth is blocked, metabolites accumulate, one (perhaps more) of which functions as a repressor of the β-galactosidase-induction system. These conclusions support the suggestion made by Neidhardt & Magasanik (1956, 1957) that glucose acted as a repressor because it was more rapidly metabolized than other carbon sources, so that, even under

growing conditions, intermediary metabolites accumulated which repressed the inducible enzymes concerned with the utilization of alternative carbon sources.

From Table 1 it is apparent that the repression produced by substances which are metabolized entirely aerobically (e.g. succinate) is reversed by DNP, whereas that produced by compounds like glucose which can also be metabolized anaerobically is not reversed. This could mean that the repressor is a product, e.g. adenosine triphosphate, common both to aerobic and anaerobic metabolism, but there is no direct evidence to support this idea.

The suggestion has been made, on the basis of genetic experiments (Pardee, Jacob & Monod, 1958), that in wild-type strains of *E. coli* β-galactosidase is inducible because the cytoplasm of the cells contains a specific repressor, whose effect upon the β-galactosidase system has to be reversed by adding an inducer if enzyme synthesis is to be obtained. In constitutive strains this repressor is assumed to be absent.

The present experiments pose the question whether the condition of blocked growth leads to the formation, from the carbon source, of more of a repressor which is assumed to be present in any case, or whether it leads to the accumulation of some other substance with the properties of a repressor. Again there is no evidence that will provide a definite answer, but the fact that repressor can apparently be produced from all carbon sources and by aerobic and anaerobic metabolism suggests that it is not as specific a substance as the repressor postulated by Pardee *et al.* (1958).

SUMMARY

1. Various strains of *Escherichia coli* deprived of ammonium salts, or of an essential amino acid or pyrimidine, are nevertheless able to produce considerable amounts of induced β-galactosidase in washed suspension.

2. Under non-growing conditions, compounds which the cells can use as sources of carbon and energy repress enzyme formation. The repressors appear to act upon the enzyme-forming system and not upon the galactoside permease.

3. Incorporation of [^{14}C]leucine into protein is unaffected under conditions which prevent β-galactosidase synthesis. Formation of this enzyme thus appears to be unreliable as a guide to the protein-forming ability of the cells.

4. The repression produced by compounds which are metabolized aerobically is reversed by 2:4-dinitrophenol; that produced by compounds metabolized anaerobically is not reversible.

5. The repressor of β-galactosidase appears to be a substance common to aerobic and anaerobic metabolism.

6. Consideration of the kinetics of enzyme synthesis shows that, in the absence of a carbon source, β-galactosidase constitutes about the same fraction of the total protein being synthesized as it does in the growing culture.

Auxotrophic mutants of *E. coli* were kindly supplied by Dr T. Alper, Dr B. D. Davis and Dr S. Spiegelman. I am much indebted to Miss R. Coyle for technical assistance.

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Studies on the Mode of Action of Excess of Vitamin A

1. EFFECT OF EXCESS OF VITAMIN A ON THE METABOLISM AND COMPOSITION OF EMBRYONIC CHICK-LIMB CARTILAGE GROWN IN ORGAN CULTURE

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Fell & Mellanby (1952) showed that in organ cultures the addition to the medium of excess of vitamin A (10–15 i.u./ml.) severely affects cartilaginous limb-bone rudiments from 6–7-day chick embryos. During 7–10 days' cultivation, the vitamin A-treated rudiments cease to grow, begin to shrink and become soft and gelatinous; the matrix no longer stains metachromatically with toluidine blue and the intercellular partitions become narrow and eventually disappear. These effects on the matrix are accompanied by cytological changes; the cartilage cells become smaller and less vacuolated and lose most of their glycogen (Fell & Thomas, 1960). They remain viable, however, and continue to multiply by mitosis. In experiments on foetal-mouse cartilage, it was shown that if the cells are killed by heating to 45° for 15 min., vitamin A has no effect on the explants (Fell & Mellanby, 1952).

In radioautographic experiments with labelled inorganic sulphate ($^{35}SO_4$), Fell, Mellanby & Pelc (1956) found that in the presence of the vitamin the explants first cease to take up sulphate and then lose that already incorporated; in the matrix the loss of $^{35}SO_4$ is accompanied by the disappearance of metachromasia. They concluded that vitamin A first inhibits the synthesis of new chondroitin sulphate and then causes the dissolution of that already formed. That these effects of vitamin A on cartilage matrix and cells are not peculiar to organ cultures has recently been demonstrated by Thomas, McCluskey, Potter & Weissmann (1960), who observed similar changes in the cartilage of young rabbits dosed with excess of vitamin A.

The object of the present investigation was to find what biochemical changes are associated with the morphological effects of excess of vitamin A on limb-bone rudiments in culture.

MATERIALS AND METHODS

Organ culture. The humeri, femora and tibiae of 7-day chick embryos were grown by the watch-glass method described by Fell & Mellanby (1952). Each watch glass contained a mixture of 15 drops of cock's blood plasma and 5 drops of embryo extract. The extract, which was made from 13- to 14-day chick embryos, was prepared by mixing equal parts of embryo pulp and Tyrode solution supplemented with 1% (w/v) of glucose; the mixture was centrifuged for 5 min.

Synthetic vitamin A alcohol (Roche Products, London) was dissolved in ethanol and added to the plasma; the same quantity (0·2%) of ethanol without the vitamin was added to the control plasma. The final medium contained 10 i.u. of vitamin A/ml. The rudiments from one side of each embryo were grown in vitamin A medium, and those from the opposite side in control medium. They were transplanted to fresh clots at 2-day intervals and were harvested for biochemical study after 6 days' cultivation.

Analyses. After incubation the bones were washed in iso-osmotic 0·9% NaCl soln., blotted dry on filter paper and weighed. The dry weights were obtained from the lipid-free material after extraction with ether. Ribonucleic acid (RNA) was estimated by the method of J. Biggers, K. Lawson, J. A. Lucy & M. Webb (unpublished), and deoxyribonucleicacid (DNA) by the method of Burton (1956). Results are expressed as μg. of nucleic acid phosphorus.

Bone rudiments and samples for hexosamine analysis were hydrolysed in 5N-HCl for 4 hr. at 100°. This procedure was always used in all analyses; no attempt was made to determine the hydrolysis conditions for optimum yields of hexosamine since the experiments were comparative and absolute quantities were not required. After hydrolysis, the samples were dried over phosphorus pentoxide *in vacuo*, and subsequently redissolved in 0·5N-HCl before analysis for total hexosamine content. Each sample was transferred to a 23 cm. column of Amberlite ion-exchange resin, CG-120 (II), washed in with 10 ml. of water and then eluted with 2N-HCl at room temperature to remove non-hexosamine components that interfere in the Elson–Morgan reaction (Boas, 1953). In experiments in which glucosamine and galactosamine were determined separately, the hydrolysed sample was redissolved in 0·3N-HCl and placed on a 60 cm. column of the same resin; elution was with 0·3N-HCl at room temperature (Gardell, 1953). The eluted hexosamines were estimated by the procedure of Rondle & Morgan (1955); four different concentrations of a standard solution of glucosamine hydrochloride were included with each batch of samples.

Metabolism. The oxygen uptake of the bones was measured by conventional Warburg techniques. Twelve bone rudiments were put into each flask. The vitamin-treated bones were each approx. 4–5 mm. in length and weighed about 2 mg. The control bones were about 6–7 mm. in length and weighed about 4 mg. The suspending medium

* Foulerton Research Fellow, Royal Society.

was Krebs–Ringer phosphate (Umbreit, Burris & Stauffer, 1957) containing 1·8 mg. of glucose/ml. and 10% of horse serum. To prevent the bicarbonate in the serum from altering the pH of the medium, the horse serum was pre-treated by the method of Friend & Hastings (1940). The gas phase was air, and 20% sodium hydroxide was included in the centre well. All flasks had an equilibration period of 10 min. at 37°, after which incubation was continued for 90 min.; over this period the rate of oxygen uptake was linear. Agitation rate was 120 strokes/min. At the end of the incubation period 1 ml. of suspending fluid was removed, made up to 7 ml. and 1·5 ml. of 5% (w/v) $ZnSO_4,6H_2O$ and 1·5 ml. of approx. 0·3N-$Ba(OH)_2$ were added. Lactic acid was estimated by the method of Barker & Summerson (1941) and glucose by the method of Nelson (1944).

Clot lysis. At the end of the culture period the bone rudiments were removed from their supporting clot and washed in salt solution. The washings were returned to the culture medium and an equal volume of 10% trichloroacetic acid was added. The precipitate was spun off and washed twice with 5% trichloroacetic acid. The acid-insoluble material was dissolved in 5N-KOH and samples of this and of the acid-soluble material were estimated for nitrogen content by the micro-Kjeldahl method (Hawk, Oser & Summerson, 1952). The amount of clot digestion is expressed as the percentage of acid-soluble nitrogen.

RESULTS

Effect of vitamin A on the composition of bone rudiments. Cultivation of the bone rudiments in the presence of added vitamin A, under the conditions reported here, severely decreased the metachromatic staining reaction of the matrix with toluidene blue. This reaction is believed to be specific for sulphate esters of high-molecular-weight substances (Kent & Whitehouse, 1955) and it is probable therefore that the lack of metachromatic staining is related to the 50% loss of amino sugars (Table 1). There was some alteration in the ratio of glucosamine/galactosamine, since the vitamin A-treated explants lost slightly more galactosamine. The decrease in total hexosamine content of the vitamin A-treated rudiments was accompanied by a quantitatively similar decrease in the wet and dry weights; that these changes were not due simply to the presence of fewer cells in the vitamin A-treated explants is shown by the fact that the ratio dry weight/DNA, which gives an approximate measure of the ratio of total mass to cell number, was much lower in the vitamin A-treated rudiments.

Though there is little cellular degeneration in vitamin A-treated explants at the stage used in this work, the vitamin affects the ratio DNA/RNA (Table 2); DNA showed a drop of 20% as compared with the controls, whereas RNA was lowered by over 60%.

Metabolism. The presentation of metabolic results is made difficult by the changes in composition of the rudiments. The severe effect of vitamin A on both the wet and dry weights pre-

Table 1. *Weight and amino sugar composition of cartilaginous limb-bone rudiments grown in culture*

The wet-wt. and dry-wt. results are the means of four experiments; analyses were made of material from 12 rudiments. The material for the amino sugar analyses was obtained from 18 rudiments.

	Wet wt. (mg./bone)	Dry wt. (mg./bone)	Dry wt./ DNA P	Total amino sugars (μg./bone)	Glucos-amine (μg./bone)	Galactos-amine (μg./bone)
Vitamin A explants	1·89	0·21	360	8·19	1·94	6·25
Control explants	4·21	0·41	570	16·92	2·92	14·0

Table 2. *Nucleic acid content of cultured limb-bone rudiments*

Results are the means of three experiments. In each experiment analyses were made of material from 12 bones.

	DNA P (μg./bone)	RNA P (μg./bone)	RNA P/ DNA P
Vitamin A explants	0·60	0·69	1·15
Control explants	0·76	1·83	2·41

Table 3. *Aerobic metabolism of cultured limb-bone rudiments*

Results are the means of three experiments. In each experiment analyses of the suspending fluid were made after 90 min. incubation in Warburg flasks at 37°. Gas phase, air; medium, Krebs–Ringer phosphate containing 10% of neutralized horse serum. Glucose concentration, 1·8 mg./ml.

	Uptake of O_2		Production of lactic acid		Utilization of glucose	
	μl./hr./bone	μl./hr./μg. of DNA P	μg./hr./bone	μg./hr./μg. of DNA P	μg./hr./ bone	μg./hr./μg. of DNA P
Vitamin A explants	1·00	1·76	16·5	27·6	24	37
Control explants	1·72	2·38	13·2	17·4	46	58

cludes their use as a basis for expressing the metabolism of the rudiments. We have adopted two criteria: the DNA phosphorus to give a measure of the metabolism on a 'cellular' basis and the metabolism of the whole rudiment without taking into account any changes in composition. The oxygen uptake of the rudiments grown in the presence of the vitamin and expressed on either basis was depressed, but the rate of lactic acid formation was increased. Glucose utilization was less in the vitamin A-treated material than in the controls (Table 3).

Clot lysis. The bone rudiments are cultivated on a plasma : embryo extract clot and are transferred to fresh medium at 2-day intervals; during each 48 hr. period the explants become surrounded by a zone of lysis, which is very much larger in the vitamin A-treated cultures than in the controls. In an attempt to measure this lytic effect we estimated the amount of acid-soluble and acid-insoluble nitrogen in the medium at the end of the experiment. After 6 days' cultivation the percentage of acid-soluble nitrogen in the control cultures was 2·25 and in the vitamin A-treated cultures 5·40. It is known that the fibrin of plasma clots may contain adsorbed fibrinolysin (Bidwell, 1953) and it was possible that the action of the vitamin was to activate this enzyme. To test this possibility, culture dishes containing vitamin A or control medium without explants were prepared and incubated for 2 days; there was no apparent lysis and no difference in the ratio of acid-soluble to acid-insoluble nitrogen between the vitamin A clots and the controls.

DISCUSSION

Although the wet and dry weights of the vitamin A-treated explants are only 50 % of the control values (Table 1), the DNA content, which may be taken as an approximate measure of cell number, is 80 % of the controls (Table 2). This suggests that the severe inhibition of growth in length and weight is mainly due to a substantial reduction in the amount of intercellular material rather than in number of cells, an interpretation that is supported by the histological finding (Fell & Mellanby, 1952) that in the vitamin A-treated cartilage the intercellular partitions become narrow and lose their metachromasia. The histological changes in the matrix are probably correlated with the low amino sugar content of the vitamin A-treated cartilage as compared with that of the controls (Table 1).

Fell *et al.* (1956) have shown that the synthesis of new cartilage matrix is inhibited by excess of vitamin A. They suggested that, under the influence of excess of vitamin A, the cartilage cells may produce an enzyme which results in the liberation of sulphated mucopolysaccharide, and hence in the dissolution of existing matrix. That hydrolytic enzyme activity might be concerned in the changes produced in cartilage matrix under the influence of excess of vitamin A was further indicated by the similarity between the effects on the intercellular material of the vitamin and of papain protease; this was true whether the comparison were made on the cartilage of young rabbits *in vivo* (Thomas *et al.* 1960) or on limb-bone rudiments in organ culture (Fell & Thomas, 1960). As a tentative working hypothesis, Fell & Thomas (1960) suggested that 'vitamin A may enhance the activity of a number of cellular enzymes, one of which resembles papain in its effect; such an enhanced hydrolytic activity might be due to the activation of enzymes, to their greater production or to their increased liberation through an increased permeability of the cells or their organelles'.

Experiments which will be reported in the following paper (Lucy, Dingle & Fell, 1961) have shown that the cells of normal embryonic cartilage in fact contain a hydrolase which under appropriate conditions can remove from the matrix most of the amino sugars and the capacity for metachromatic staining. Another paper (Dingle, 1961) suggests that the vitamin acts by releasing enzymes from the lysosomes.

SUMMARY

1. After 6 days' cultivation in medium containing added vitamin A (10 i.u./ml.) the wet and dry weights and amino sugar content of cartilaginous limb-bone (chick) rudiments were half those of controls on normal medium.

2. Compared with the control cultures, the amount of deoxyribonucleic acid was 80 % and the amount of ribonucleic acid was 40 %.

3. The vitamin depressed the rate of oxygen uptake but increased that of lactic acid formation.

4. There was twice as much acid-soluble nitrogen in the used culture medium (plasma–embryo extract clot) of the vitamin A-treated explants as in that of the control, indicating a greater proteolytic activity in the presence of the vitamin.

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Studies on the Mode of Action of Excess of Vitamin A

2. A POSSIBLE ROLE OF INTRACELLULAR PROTEASES IN THE DEGRADATION OF CARTILAGE MATRIX

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When cartilaginous limb-bone rudiments from 6- to 7-day chick embryos are cultivated *in vitro*, the cartilage differentiates into a hypertrophic region, proliferative zones of flattened cells and epiphyses as in normal development. If excess of vitamin A is added to the culture medium, the cartilage differentiates at approximately the normal rate but the metachromatic staining properties of the matrix are greatly reduced (Fell & Mellanby, 1952). A similar loss of metachromasia occurs when papain is added to the culture medium (Fell & Thomas, 1960). The injection of papain into rabbits results in a collapse of the ears, accompanied by disappearance of metachromasia from the matrix of the ear cartilage (Thomas, 1956). Similar injections can also cause total disappearance throughout the body of the metachromatic staining properties of cartilage matrix (Spicer & Bryant, 1958; McCluskey & Thomas, 1958) and the appearance in blood and urine of a mucopolysaccharide that resembles chondroitin sulphates A and C (Bryant, Leder & Stetten, 1958). A depletion of the chondromucoprotein of cartilage and decrease of ^{35}S content of cartilage matrix have been observed (Tsaltas, 1958) in rabbits which have received sodium [^{35}S]sulphate and papain; later an increased quantity of ^{35}S in serum and urine and of glucuronic acid in urine occur. The administration of large amounts of vitamin A to rabbits causes histological changes in cartilage that are remarkably similar to those seen in animals treated with small amounts of papain (Thomas, McCluskey, Potter & Weissmann, 1960).

These observations suggest that some of the effect of vitamin A on the cartilage of embryonic-chick rudiments may be mediated through the action of a proteolytic enzyme system present in the cartilage, which degrades the structural components of the matrix with a resultant loss of polysaccharide. In the preceding paper it is reported that, when grown *in vitro* in the presence of excess of vitamin A, the cartilaginous limb-bone rudiments from 6- to 7-day chick embryos have a greater proteolytic effect on the medium than control explants in normal medium (Dingle, Lucy & Fell, 1961). A possible interpretation of this is that the activity of proteolytic enzymes in the tissue is enhanced by vitamin A. In this paper evidence is reported that an enzyme system which will degrade cartilage matrix is present in bone rudiments from normal chick embryos. The enzyme, or group of enzymes, is active after hypo-osmotic treatment of the tissues; the optimum pH of the system is in the acid region.

MATERIALS AND METHODS

Material. All experiments were performed on limb-bone rudiments from 8–9-day chick embryos. At this stage the limb bones consist of a cartilaginous rod differentiated into the usual three zones of epiphyses, flattened (proliferative) cells and hypertrophic cells; the hypertrophic zone is

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covered by a layer of periosteal bone. Embryos of the same age vary considerably in their degree of development; in particular, embryos taken in summer are usually larger and more advanced than those obtained in winter. The rudiments (cartilage, periosteal bone and periosteum) were dissected as free as possible of muscle and connective tissue and were kept at room temperature, in a mixture of equal parts of iso-osmotic salt solution and amniotic fluid, until the beginning of the experiment. Before the rudiments were weighed, excess of fluid was removed with filter paper.

Histology. At the end of the experimental period, bone rudiments were fixed in 3% acetic acid–Zenker's fluid for 30 min. followed by Zenker's fluid without acetic acid for not less than 3 hr. After being washed, dehydrated and cleared in cedarwood oil, the rudiments were embedded in paraffin wax and serially sectioned. Sections were stained for about 30 min. in 0·5% of toluidine blue (G. T. Gurr Ltd., London) in 5% ethanol, rinsed in tap water, dehydrated, cleared and mounted in DePeX (G. T. Gurr Ltd.), in which the metachromatic staining is preserved for years.

In the experiments in which the effect of pH on loss of hexosamine was investigated, 36 limb-bone rudiments from three 9-day eggs were used at each pH. One rudiment was removed from each tube after the bones had been incubated in buffer for 2 hr., and was fixed for histological examination. The error in the estimation of the total hexosamine content of the treated bones caused by the removal of the bone rudiment would be very small, and no allowance was made for this in the determination of the percentage of hexosamine liberated into the buffer.

Effect of pH *on protein breakdown.* Freshly dissected bone rudiments were placed in water at 4° for 1 hr.; they were then incubated for 2 hr. at 37° in 0·1 M-sodium acetate–HCl buffer (Vogel, 1948) for pH values 1–5. For pH values 6–8, 0·1 M-sodium acetate was adjusted to the desired pH by the addition of 0·1 N-NaOH. All pH values were checked, both before and after the incubation, by means of a glass electrode. Two tibiae and two femora were used at each pH. After incubation the supernatant solution was removed from each tube and the bones were washed with several small quantities of buffer. The buffer solutions were dried *in vacuo* over P_2O_5 and KOH. Each extract was hydrolysed for 24 hr. at 110° in HCl of constant boiling point; the residual rudiments were hydrolysed similarly. The hydrolysates were dried *in vacuo* over P_2O_5 and KOH; the hydrolysed material was redissolved in a suitable portion of water and analysed for total α-amino nitrogen with the KCN–ninhydrin reagent of Yemm & Cocking (1955) after the addition of N-sodium acetate buffer, pH 5·1 (1 ml.) (Moore & Stein, 1954). Leucine was used as the standard.

Fractionation of protein and determination of protein breakdown in rudiments. Freshly dissected bones from 9-day eggs were placed in water at 4° for 1 hr. and were then incubated for 2 hr. at 37° in 2 ml. of 0·1 M-sodium acetate solutions of different pH values (pH 1–8). Three metatarsals, one femur and one tibia were incubated at each pH. The supernatant buffer was removed from each tube and the bones were washed with several small quantities of buffer; the washings were added to the main supernatant. An equal volume of 10% trichloroacetic acid was added to each buffer supernatant and the solution allowed to stand for 2 hr. in the cold before centrifuging. The scanty, barely detectable precipitates were discarded. Each solution was dried *in vacuo* over P_2O_5 and KOH.

The treated bone rudiments were extracted twice in the cold with two portions (1 ml.) of 5% trichloroacetic acid (10 min. periods), followed by three 15 min. extractions with 5% trichloroacetic acid at 90°. The cold and hot trichloroacetic acid extracts were dried *in vacuo* over P_2O_5 and KOH.

Each of the dried samples described above was hydrolysed in HCl of constant boiling point (2 ml.) for 24 hr., in a sealed tube at 110°; the residual bone rudiments were hydrolysed similarly. The hydrolysates were dried *in vacuo* over P_2O_5 and KOH; final traces of acid were removed by drying under an infrared lamp. Each hydrolysate was redissolved in a suitable volume of water and duplicate portions (2 ml.) were analysed for total α-amino nitrogen as described above.

Amino acid analysis of degraded-protein components. Limb-bone rudiments (354 mg. wet wt.) from sixteen 9-day eggs were placed in water at 4° for 1 hr. and then incubated at 37° for 2 hr. in 0·1 M-sodium acetate buffer (4 ml.) at pH 4. The buffer solution was removed and the rudiments were washed with several small quantities of buffer; the washings were added to the main supernatant buffer. An equal volume of 12% trichloroacetic acid was added to the buffer solution. Although the buffer contained a few small pieces of detached tissue, there was no apparent increase in turbidity after the addition of the acid. The solution was centrifuged and the scanty residue discarded. The supernatant solution was dialysed in the cold for 36 hr. against five successive portions (25 ml.) of distilled water. After the total diffusate (125 ml.) had been reduced to a small volume in a rotary evaporator, the solution was heated for 1 hr. at 100° in the presence of a small piece of porous pot to decompose the trichloroacetic acid. The diffusate and the non-dialysable material were dried over P_2O_5 *in vacuo* and then hydrolysed in HCl of constant boiling point (2 ml.) for 24 hr. under N_2 at 110°. Both hydrolysates were dried over P_2O_5 and KOH *in vacuo*, then redissolved in water, and portions analysed for total α-amino nitrogen with ninhydrin, leucine being used as a standard.

A portion of the hydrolysate of the non-dialysable material was applied to a 150 cm. column of Amberlite resin, CG-120 (II), on which the neutral and acidic amino acids were separated by the procedure of Moore, Spackman & Stein (1958). The amino acids in the eluate were quantitatively determined with the KCN–ninhydrin reagent of Yemm & Cocking (1955). Proline was determined in the eluate with the acid–ninhydrin reagent of Chinard (1952), and hydroxyproline was estimated in the eluate by a modification (Lawson & Lucy, 1960) of Neuman & Logan's (1950) procedure in which 1% hydrogen peroxide was used and colour developed at 60–65°.

A portion of the hydrolysed diffusate was de-ionized by passage through a column of Dowex 50 in the H^+ form in order to remove the Na^+ ions present in this fraction from the acetate buffer, pH 4. The solution was then chromatographed on Whatman no. 54 paper in a solvent system of phenol–water–ammonia with an ammonia atmosphere. The chromatogram was run for 10 hr. at room temperature, dried by heat and developed by ninhydrin.

Release of proteolytic activity. The bone rudiments from 16 eggs were placed in water at 4° for 1 hr.; after removal of the rudiments the solution was centrifuged to eliminate any

debris and the proteolytic activity of the supernatant estimated.

Separation of intracellular fractions. The bone rudiments were homogenized in 0·25M-sucrose at 4° and the homogenate was centrifuged in a Serval Superspeed centrifuge at 4°. The first fraction, consisting mainly of nuclei and debris (Schneider & Hogeboom, 1950), was obtained after 5 min. at 700 *g*. The pellet was washed with sucrose and the supernatant and washings were centrifuged at 10 000 *g* for 20 min. The proteolytic activity of the 'nuclear' fraction and of the 'mitochondrial' fraction was measured after the pellets had been homogenized in water. In the supernatant fraction, which even after dilution contained a considerable concentration of sucrose, the solutions were kept cold to avoid hydrolysis of the sugar by the trichloroacetic acid (Gianetto & de Duve, 1955).

Estimation of proteolytic activity. Proteolytic activity was measured by the method of Anson (1938). Bovine haemoglobin enzyme–substrate (Armour Laboratories) was used at a final concentration of 2% (w/v). The enzyme activity was measured at 37° in 0·1M-sodium acetate buffer and expressed as the increase (μg.) in acid-soluble 'tyrosine'.

Estimation of acid mucopolysaccharide. After 1 hr. in water at 4° the bone rudiments were incubated at pH 4 in acetate buffer at 37° for 2 hr. The bones were then removed, the fluid was cleared of debris by centrifuging and polymeric acid polysaccharide estimated by the turbidimetric method of Di Ferrante (1956). The buffer extract was adjusted to pH 6 and 1 ml. was treated with 2·0 ml. of 2·5% cetyltrimethylammonium bromide in 2% NaOH. After 5 min. at 37° the extinction was read at 400 mμ. Dilutions of a standard solution of chrondoitin sulphate L. Light and Co. Ltd.) were made at the time of each set of estimations; the method gave a linear relationship between extinction and polysaccharide concentration over the range 20–180 μg./ml.

The mucopolysaccharide in the 0·1M-sodium acetate buffer extract was treated with testicular hyaluronidase (Benger Laboratories Ltd.), the pH adjusted to 6·4 by the addition of 0·1N-NaOH, and the extract then incubated at 37°. The remaining polymeric material was estimated turbidimetrically.

RESULTS

Loss of amino sugars from rudiments during treatment with papain. Fell & Thomas (1960) have shown that incubation in a solution of papain protease destroys metachromasia in chick-limb-bone rudiments. As shown in Table 1, the enzyme also causes a rapid release of hexosamine from the rudiments. This finding prompted an investigation into other methods of removing extracellular material from embryonic limb cartilage.

Effect of hypo-osmoticity on metachromasia of cartilage matrix. Initial experiments were made to see if the effect of treatment with excess of vitamin A upon the metachromatic staining could be simulated by extraction of the polysaccharide present in the cartilage. A number of salt solutions that are commonly used to remove chondroitin sulphate from cartilage were tried. The rudiments were extracted for 1–24 hr. with the following solutions: alkaline KCl (Einbinder & Schubert, 1950), neutral $CaCl_2$ (Blix & Snellman, 1945) and water (Shatton & Schubert, 1954). The alkaline reagent had almost no action upon the metachromatic staining properties of the matrix and the neutral salt solution also was ineffective except in the 24 hr. experiment. The water-treatment, however, decreased metachromasia of the cartilage after 3 hr., indicating a loss or depolymerization of sulphated mucopolysaccharide. As is shown in Table 2, water was more effective at 37° than at 4°, whereas incubation at 37° in alkaline KCl had no effect.

Effect of pH *on loss of metachromasia.* After immersion of the rudiments in water at 4° for 1 hr., the chondrocytes became shrunken and their nuclei pycnotic; there was no obvious effect on the metachromatic staining of the matrix. In the following experiments this procedure was adopted as a standard hypo-osmotic pretreatment with the object of disrupting the cells and their organelles and thus releasing the intracellular hydrolytic enzymes.

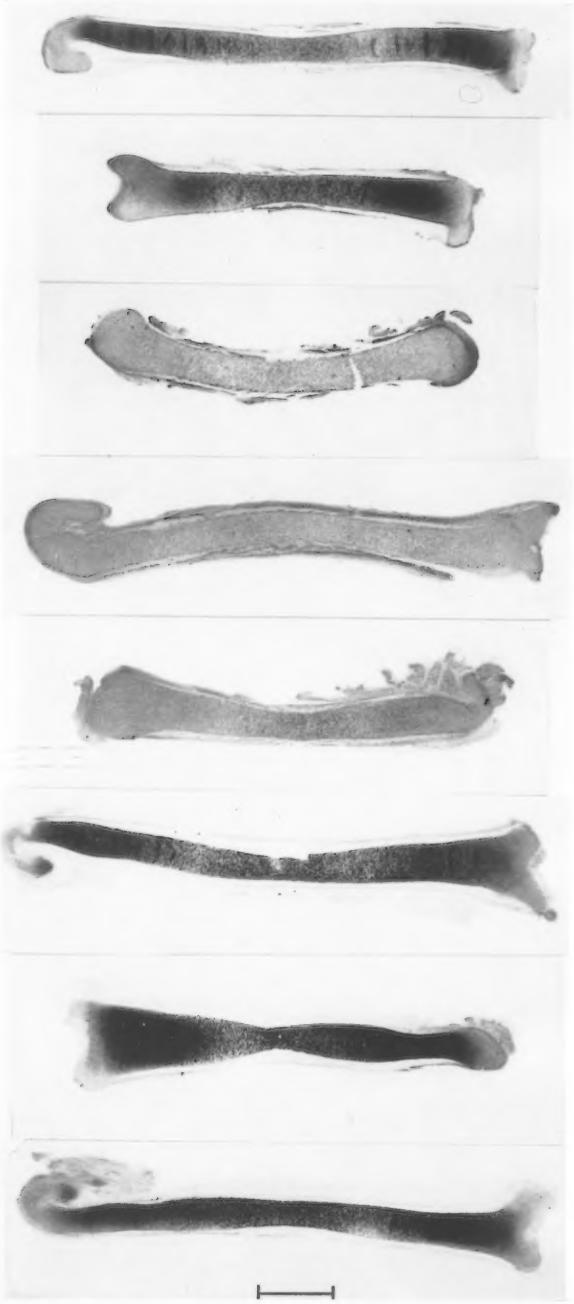
Two more experiments were made (Plate 1 and Table 3) in which the bones, after the usual hypo-osmotic pretreatment, were incubated in buffer solutions of pH from 1 to 8. The greatest loss of metachromasia was at pH 3 and 4, and there was little (Expt. 314) or no (Expt. 292) obvious effect below pH 3 or above pH 5. The rudiments in Expt. 314 were at a more advanced stage than those of Expt. 292 and show an apparently greater histological response; this may be due to the fact that in normal development the matrix becomes less intensely metachromatic with age. In both experiments, however, maximum loss of metachromasia was between pH 3 and 4.

Relationship between loss of metachromasia and loss of hexosamines. At pH 4 and 5 more than half of the hexosamine content of the rudiments was liberated into the medium during the 2 hr. incubation period after the hypo-osmotic treatment (Fig. 1). In this experiment (Expt. 314 in Table 3, and Plate 1) parallel observations were made on the disappearance of metachromasia and on the loss of hexosamines; the results showed that the two phenomena had the same pH dependence.

The total quantity of amino sugars liberated during a 4 hr. incubation period at pH 3 was no

EXPLANATION OF PLATE 1

Sections of limb-bone rudiments from 9-day chick embryos, incubated at 37° in buffers of different pH values after 1 hr. in water at 4°. The sections are stained with toluidine blue; note complete loss of metachromatic staining of the cartilage matrix at pH 3 and 4 but little effect on the metachromasia at pH 1, 2, 6, 7 and 8.



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greater than the amount removed during 2 hr. This was at first thought to indicate that a specific polysaccharide fraction was completely liberated within 2 hr. Analyses showed, however, that the pH 3 buffer contained both glucosamine and galactosamine after incubation for 2 hr.; in the buffer, glucosamine represented 19% of the total hexosamine present and in the residual bone rudiments it accounted for 12% of the total. Since these proportions were not very different it seemed more likely that, if an enzyme caused the observed effects, denaturation of the enzyme at pH 3 was responsible for cessation of liberation of hexosamine within 2 hr.

Loss of high-molecular-weight polysaccharide. In the above-described experiments, loss of hexosamine from the rudiments was estimated after acid hydrolysis. To find whether the polysaccharide liberated at pH 4 after hypo-osmotic treatment of the rudiments was of high molecular weight or

Table 1. *Effect of papain on the hexosamine content of 8-day chick-limb-bone rudiments*

Bone rudiments were incubated at 35° for 2 hr. in Tyrode's solution containing 20 μg. of activated papain/ml.

Tissue	Fraction	Hexosamine content (μg.)	Wet. wt. of tissue after treatment (mg.)
Papain-treated (cartilage from one side of two embryos)	Tyrode's solution containing papain	230	—
	Insoluble bone residue	Trace	26
Control (cartilage from other side of two embryos)	Tyrode's solution	Nil	—
	Insoluble bone residue	262	48

Table 2. *Effect of hypo-osmotic treatment on metachromasia from cartilage matrix of 9-day rudiments*

The degree to which the various treatments affected the metachromatic staining of the cartilage matrix is roughly indicated by the following code: ?+, possibly a slight effect; +, metachromasia largely gone from the cartilage immediately beneath the articular surface; + +, general loss from the epiphyses; + + +, loss from epiphyses + proliferative zones of flattened cells; + + + +, almost complete disappearance of metachromasia throughout rudiment; + + + + +, complete disappearance.

Treatment	Temperature	Length of treatment (hr.)	Bone rudiment	Effect on metachromasia
Distilled water	4°	3	Femur	Nil
			Femur	+
	4	6	Femur	Nil
			Humerus	Nil
	37	3	Tibia	+ + +
			Humerus	+ +
	37	6	Femur	+ + + +
			Tibia	+ + + +
30% KCl containing 1% of K_2CO_3	37	3	Tibia	Nil
			Humerus	Nil

Table 3. *Effect of* pH *on loss of metachromasia from cartilage matrix of 9-day rudiments*

All rudiments were pretreated for 1 hr. with distilled water at 4°, then incubated in buffer at 37·0° for 2 hr. For the meaning of +, etc., see Table 2.

	Expt. 292		Expt. 314	
pH	Bone	Effect on metachromasia	Bone	Effect on metachromasia
1·1	Femur	Nil	Tibia	+
2·0	Femur	Nil	Femur	+
3·1	Femur	–	Femur	+ + + + +
3·1	Metatarsal	+ + +	–	–
4·0	Femur	+ + + +	Tibia	+ + + + +
5·0	Femur	+ +	Tibia	+ + + +
6·0	Femur	+	Tibia	?+
7·0	Femur	Nil	Femur	?+
8·0	Femur	Nil	Tibia	?+

composed of degraded smaller molecules, the buffer extracts were examined by the Di Ferrante (1956) method at the end of the 2 hr. incubation period. Expressed as chondroitin sulphate, the amount of acid polysaccharide was very similar to that expected from the estimation of amino sugar. Treatment of the buffer extract with testicular hyaluronidase for 15 min. at 37° caused a 32% drop in turbidity, and after longer incubation the turbidity disappeared completely.

The turbidimetric method was also used to follow the rate of release of polysaccharide from the bone rudiments in buffer at pH 4 (Fig. 2). It is interesting to compare the rate of a release with the rate of loss of metachromatic staining. After 10 min. metachromasia had largely gone from the epiphyses; after 20 min. it had disappeared from the proliferative zones also and after 50 min. the matrix was unstained throughout the rudiment; less than a quarter of the polysaccharide was extracted during this period. This result is similar to the observation mentioned in a previous section, namely, that after 2 hr. incubation at pH 4, only 50% of the hexosamine had been removed although metachromasia had completely disappeared.

Loss of protein. Table 4 shows that there is maximum release of ninhydrin-positive material into the buffer at pH 4–5. At pH 7, 12·8% of the total ninhydrin-positive material in the rudiment was extracted and 21·2% at pH 5; this contrasts with 4% of the amino sugars removed at neutrality and 53% at pH 5 (Fig. 1).

In a further experiment rudiments were again treated with water at 4° and then incubated at 37° for 2 hr. in 0·1M-buffers of differing pH. The trichloroacetic acid-soluble material in the buffers was estimated with ninhydrin after acid hydrolysis; similar estimations were made on three fractions from the rudiments: material soluble in cold 5% trichloroacetic acid, that soluble in hot 5% trichloroacetic acid, and the acid-insoluble fraction.

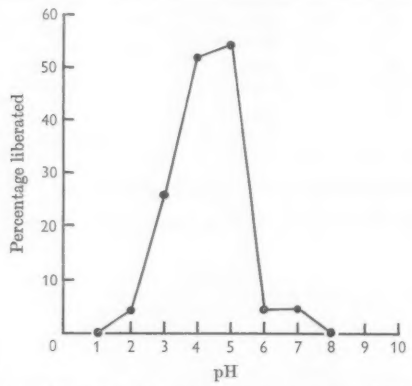

Fig. 1. Liberation of hexosamine from bone rudiments. Bone rudiments were treated with water at 4° for 1 hr. and then incubated in 0·1M-sodium acetate buffers of different pH values for 2 hr. at 37°. The hexosamine in the buffer and that remaining in the rudiments was estimated as indicated in the text.

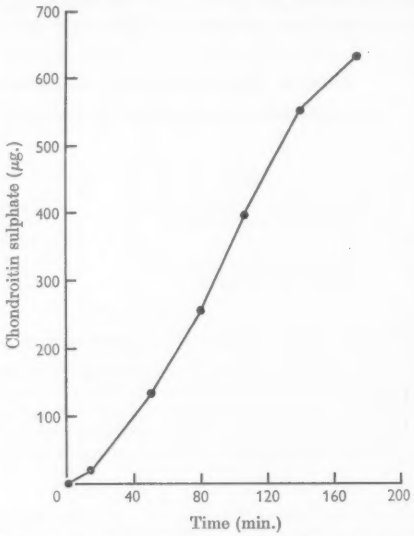

Fig. 2. Rate of polysaccharide release from bone rudiments. Bone rudiments were treated with water at 4° for 1 hr. and then incubated in 0·1M-sodium acetate buffer, pH 4·0, at 37°. The polysaccharide was measured turbidimetrically (in the buffer) by the addition of cetyltrimethylammonium bromide dissolved in 2% NaOH. Results are expressed in μg. of chondroitin sulphate.

Table 4. *Effect of* pH *upon the loss of ninhydrin-positive material from bone rudiments*

After the standard pretreatment with water the rudiments were incubated in acetate buffers of different pH values. The ninhydrin-positive material in the buffer and that remaining in the rudiments was hydrolysed as described in the text. Results are expressed as μg. of leucine equivalents.

	Ninhydrin-positive material		
pH	In buffer (μg.)	In rudiments (μg.)	Percentage extracted
1·1	95·1	405	19·0
2·0	83·5	503	14·2
3·1	110·0	497	18·1
4·0	131·0	524	20·0
5·0	113·0	418	21·2
6·0	104·0	529	16·4
7·0	65·0	442	12·8
8·0	88·8	553	13·8

It has been shown by Fitch, Harkness & Harkness (1955) that extraction of tissues with hot trichloroacetic acid removes collagen almost completely; it has been confirmed in this laboratory that the hot trichloroacetic acid extracts contain most of the hydroxyproline of embryonic chick-limb-bone rudiments (J. D. Biggers, K. Lawson, J. A. Lucy & M. Webb, unpublished observations).

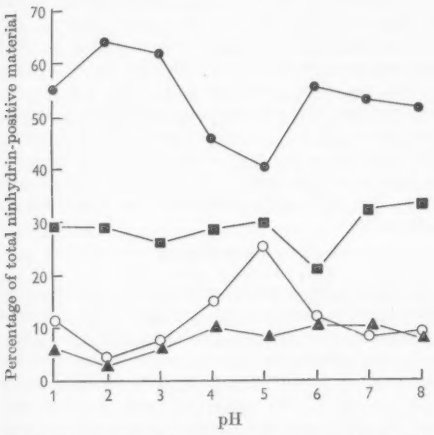

Fig. 3. Distribution of ninhydrin-positive material. Bone rudiments were treated with water at 4° for 1 hr. and then incubated in 0·1 M-sodium acetate buffers of different pH values for 2 hr. at 37°. The rudiments were washed and extracted first with cold 5% trichloroacetic acid and then with hot 5% acid. The ninhydrin-positive material was estimated, after hydrolysis, in these two fractions, in the acid-insoluble material and also in the acid-soluble material in the buffer. ●, Trichloroacetic acid-insoluble; ○, buffer extract (acid-soluble); ■, hot-acid-soluble; ▲, cold-acid-soluble.

In the present experiments there was significant proteolysis in the region pH 4–5. At these pH values, corresponding to those at which polysaccharide is lost, there was a reduction in the content of trichloroacetic acid-insoluble protein of the treated rudiments and an increase in the trichloroacetic acid-soluble material that was liberated into the buffer solution (Fig. 3). By calculation from the data of experiments described above, it was found that hexosamine liberated into the buffer at pH 5 could not account for more than approximately 15% of the ninhydrin-positive material in the buffer; even less hexosamine was probably present, owing to the destruction of amino sugars that occurs during hydrolysis in 6 N-HCl at 110° for 24 hr.

In a subsequent experiment, bone rudiments were incubated at pH 4 for 2 hr., after the usual hypo-osmotic pretreatment. Although the amino acid-containing material liberated into the buffer was soluble in cold 5% trichloroacetic acid, more than 80% of the ninhydrin-positive material (estimated after 24 hr. acid hydrolysis) was non-dialysable. It was thought that either the molecular weight of the material was too low for it to be precipitated by trichloroacetic acid but large enough for the material to be non-diffusible or that the material was attached to a relatively large proportion of non-diffusible polysaccharide. Amino acid analysis of the non-dialysable protein fraction showed that it was unlikely to have been derived from collagen since it contained a low proportion of glycine and very little hydroxyproline; the analysis supported the observations described above, which indicated that the protein component liberated into the buffer was derived from the non-collagenous protein of the rudiments. The partial amino acid analysis of the non-dialysable fraction liberated into the pH 4 buffer is shown in Table 5, in comparison

Table 5. *Partial amino acid composition of non-dialysable material liberated from rudiments at* pH 4

Isolation and analysis of this material was as described in the text. Figures in column 3 are the unpublished results of J. D. Biggers, K. Lawson, J. A. Lucy & M. Webb.

	Non-dialysable fraction in buffer, pH 4		Non-collagenous protein from 7-day chick tibia (molar ratios, glycine = 1·00)	Human-bone collagen (molar ratios, glycine = 1·00 Eastoe, 1955)
Amino acid	g. of N/100 g. of α-amino N	Molar ratios (glycine = 1·00)		
Glycine	6·75	1·00	1·00	1·00
Hydroxyproline	0·29	0·04	0·004	0·31
Aspartic acid	4·60	0·68	1·21	0·15
Threonine	5·02	0·74	0·67	0·06
Serine	6·33	0·94	0·77	0·11
Glutamic acid	5·07	0·75	1·62	0·23
Proline	6·58	0·97	0·74	0·39
Alanine	5·41	0·80	0·98	0·36
Valine	3·19	0·47	0·63	0·07
Methionine	0·61	0·09	0·21	0·02
Isoleucine	1·80	0·27	0·54	0·04
Leucine	2·94	0·44	0·97	0·08

with the composition of the protein that is insoluble in hot trichloroacetic acid obtained from the tibia of the 7-day embryonic chick (J. D. Biggers, K. Lawson, J. A. Lucy & M. Webb, unpublished observations); the non-dialysable fraction had more serine and proline and much less aspartic acid, glutamic acid and leucine.

The dialysable fraction obtained in this experiment was chromatographed on paper after acid hydrolysis. Although all the ninhydrin-staining components were not identified, glutamic acid and aspartic acid were present, and glycine appeared to be absent. There was no evidence of a large quantity of hydroxyproline in the diffusible fraction.

Loss of weight. Loss of wet weight (Fig. 4) by the rudiments, during incubation in buffers of different pH, was determined at the same time as measurements were made of the protein fractions obtained by the use of trichloroacetic acid (Fig. 3). As shown in Fig. 4, the optimum pH for loss of wet weight was similar to that for hexosamines, but there was also an apparently non-specific loss of weight at very low pH values.

Proteolytic activity of the aqueous extract. Since the above-described experiments indicated that the rudiments probably contained an enzyme or enzymes that were released by hypo-osmotic treatment, an attempt was made to obtain these enzymes in soluble form.

It was found that the initial distilled-water treatment for 1 hr. at 4° released an active protease into the water. This had a pH optimum against haemoglobin of between 2 and 3 (Fig. 5); heating the extract to 100° for a few seconds resulted in a complete loss of activity at pH 4 with haemoglobin. Homogenization of the bone rudiments in water at 4° gave a much more active extract; with this method it is possible to measure the activity released from bone rudiments weighing only a few milligrams.

Proteolytic activity after homogenization in iso-osmotic sucrose. Though the proteolytic activity was easily released by hypo-osmotic treatment of the rudiments, homogenization in iso-osmotic sucrose released little. Fractionation of the sucrose homogenate by centrifuging, followed by homogenization of the fractions in water, showed that 51 % of the activity against haemoglobin at pH 4 was contained in the 'mitochondrial' fraction, 33 % in the 'nuclear' fraction and 16 % was in the supernatant. The pH optimum of all three fractions was about pH 3. It would appear that the proteolytic enzyme or enzymes of cartilage with a low pH optimum are mainly in an inactive particulate form, but are easily activated by hypo-osmotic treatment.

DISCUSSION

It has been demonstrated that treatment of chick-bone rudiments with distilled water, followed by incubation at pH 4–5, produces rapid changes in both the histological appearance and the chemical composition of the tissue. During incubation for 2 hr. at pH 4, the metachromatic staining properties of the cartilage matrix are greatly reduced,

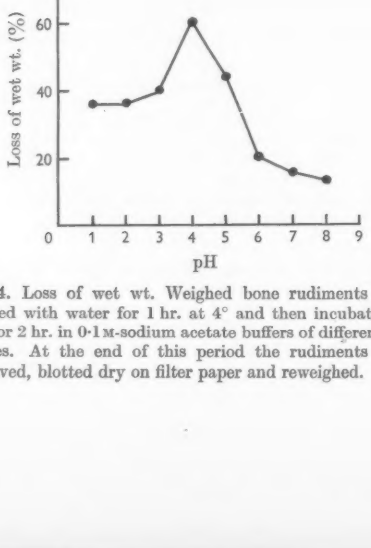

Fig. 4. Loss of wet wt. Weighed bone rudiments were treated with water for 1 hr. at 4° and then incubated at 37° for 2 hr. in 0·1 M-sodium acetate buffers of different pH values. At the end of this period the rudiments were removed, blotted dry on filter paper and reweighed.

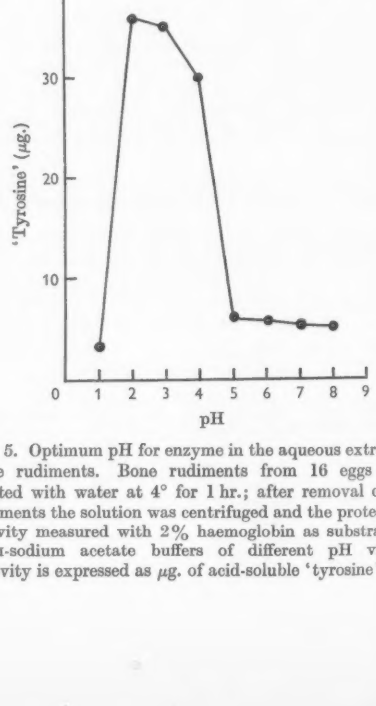

Fig. 5. Optimum pH for enzyme in the aqueous extract of bone rudiments. Bone rudiments from 16 eggs were treated with water at 4° for 1 hr.; after removal of the rudiments the solution was centrifuged and the proteolytic activity measured with 2% haemoglobin as substrate in 0·1 M-sodium acetate buffers of different pH values. Activity is expressed as μg. of acid-soluble 'tyrosine'.

and half of the hexosamine content of the rudiments is removed. That this effect is not peculiar to chick cartilage has been demonstrated in experiments, to be reported elsewhere, on late foetal-mouse bones pretreated with distilled water and then incubated in buffers of different pH; in the terminal cartilage of these bones, metachromasia was completely removed at pH 3 and 4 but was almost unchanged at pH 2 or 6.

In the chick rudiments the loss of amino sugars occurs primarily by removal of polysaccharide of high molecular weight, whereas acid-soluble products are lost into the buffer from the protein constituents of the tissue. The strong pH-dependence indicates enzymic degradation of the tissue, since it is unlikely that a chemical-extraction process would be so sharply dependent upon pH. The much more rapid loss of metachromasia in distilled water at 37° than at 4° also suggests an enzymic mechanism. Einbinder & Schubert (1950) state that enzyme action might have been responsible for the fact that only 11% of the chondroitin sulphate was extracted by potassium chloride solution from fresh wet cartilage, but 72% after storage of the wet cartilage at 0° for 4–6 weeks.

Both papain (Bryant *et al.* 1958) and plasmin (Lack & Rogers, 1958) remove polymeric polysaccharide from cartilage. In the present experiments, protein was degraded at pH 4 and 5 after hypo-osmotic treatment of the chick rudiments and it seems possible that proteolytic action was the primary cause of the loss of polymeric polysaccharide. This hypothesis is supported by the demonstration that treatment of the rudiments with water causes the release of a proteolytic enzyme, with an acid pH optimum, that is active against haemoglobin.

Although components soluble in trichloroacetic acid that were ninhydrin-positive were released from the rudiments at pH 4, more than 80% of this material was non-dialysable. The amino acid composition of the non-dialysable material indicates that it was probably derived from the non-collagenous protein of the rudiments rather than from collagen. This evidence, combined with the observed breakdown at pH 4 and 5 of the protein of the rudiments insoluble in trichloroacetic acid, shows that maximum liberation of polysaccharide was associated with breakdown of the non-collagenous protein of the tissue. It is thought that these observations may be related to those of Shatton & Schubert (1954), Partridge & Davis (1958) and Muir (1958), who found a polysaccharide–protein complex in mammalian cartilage, which was composed of a non-collagenous protein bound to chondroitin sulphate. Mathews (1956) also found a protein, in chondroitin sulphate preparations, that had a hydroxyproline content of only 0·1%; proteolytic enzymes have been shown to degrade these mucoproteins of cartilage, with a consequent loss of viscosity (Shatton & Schubert, 1954; Muir, 1958).

The amino acid-containing material liberated from the rudiments at pH 4, that was non-dialysable, differed significantly in amino acid composition from the non-collagenous protein of whole chick-limb-bone rudiments in having considerably less glutamic acid, aspartic acid and leucine, and more serine and proline relative to glycine (Table 5). On the other hand, the diffusible fraction contained glutamic acid and aspartic acid but apparently no glycine. One possible interpretation of these observations is that the degradation of the non-collagenous protein is the result of enzyme action at the linkage between glycine and glutamic acid. The non-dialysable fraction may be associated in some manner with polysaccharide in the cartilage, since at pH 4 the two components are liberated simultaneously from the bone rudiments. Further study of this system may throw light on the mode of linkage of protein and polysaccharide in cartilage, and also on the enzymic mechanism by which the polysaccharide may be liberated. More serine is present relative to glycine in the non-dialysable component than in the non-collagenous protein of whole rudiments; Muir (1958) has reported that serine was the only amino acid that was not greatly decreased by papain digestion of the polysaccharide–protein complex obtained from pig tracheal cartilage.

Gianetto & de Duve (1955) found that the cathepsin activity of rat liver is contained within cytoplasmic granules and that full cathepsin activity occurs when the enzyme is released by exposure of the lysosomes to hypo-osmotic media (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). A possible interpretation of the experiments described in this paper is that the proteolytic enzyme liberated from the cartilage cells by treatment with distilled water subsequently attacks the protein to which the polysaccharide of the cartilage is attached. The enzyme concerned may be a cathepsin, and in connexion with this suggestion, Press, Porter & Cebra (1960) have recently isolated a proteolytic enzyme from bovine spleen which has been named cathepsin D since it will not hydrolyse any of the typical substrates of cathepsins A, B and C. Cathepsin D has a pH optimum at 4·2 with denatured albumin, and at pH 3 with denatured haemoglobin; it does not require cysteine for activation.

These studies were undertaken to see if the cartilaginous limb-bone rudiments of the chick possess enzymes that are capable of producing effects similar to those observed when the rudiments are cultivated in a medium containing

excess of vitamin A. The results described show that the limb cartilage contains a proteolytic enzyme that may release polysaccharide by degradation of structural protein. The reduction of metachromatic staining and loss of hexosamines from the cartilage matrix as a result of incubation in the buffer solutions are similar to the changes observed in explants exposed to excess of vitamin A. It is probable that chondroitin sulphate is the principal polysaccharide of the cartilaginous bone rudiments and that it is freed under the conditions used, since galactosamine comprises 80% of the hexosamine liberated at pH 3. Glucosamine also is liberated at pH 3 and more than one polysaccharide must therefore be lost from the rudiments since chondroitin sulphate contains little or no glucosamine. Hypo-osmotic treatment of cartilage cells may release a number of enzymes, each of which can degrade a particular component of cartilage matrix.

Chick-limb-bone rudiments grown in culture in the presence of excess of vitamin A produce more lactic acid than the corresponding rudiments in control cultures (Dingle *et al.* 1961) and this increased acidity in the vitamin A-treated cartilage would favour degradation of the matrix, since this is much more rapid at pH 5 than at pH 7.

The results described in this paper provide some additional support both for the hypothesis (Fell & Thomas, 1960) that vitamin A acts on cartilage matrix by enhancing the enzymic activity of the chondrocytes and also for the suggestion (Dingle, 1961) that the vitamin may increase proteolytic activity by altering the permeability of the lysosomes. Further investigations are needed, however, to isolate and characterize the enzyme or enzymes responsible for the changes in the rudiments at pH 4, and to find whether these changes are in fact directly related to those produced by excess of vitamin A.

SUMMARY

Experiments were made to find whether normal cartilage contains an enzyme that is capable of producing an effect on the matrix like that of vitamin A.

2. Normal cartilaginous rudiments were pretreated with distilled water to disrupt the cells and their organelles and then incubated in buffer solutions of pH 1–8. Between pH 3 and 5 the metachromatic staining of the matrix was greatly reduced, half the hexosamine content was lost (mostly as polysaccharide of high molecular weight) and protein components were liberated into the buffer. Above and below pH 3–5 there was little effect on the cartilage matrix.

3. An extract having proteolytic activity at a pH optimum of 3 has been obtained from cartilage. The proteolytic activity is located in cytoplasmic particles, from which it can be liberated by water; it is destroyed by heating at 100°.

4. It is concluded that normal chondrocytes contain an enzyme or a group of enzymes capable of producing an effect on cartilage matrix that closely resembles that produced by excess of vitamin A.

We are indebted to Dr M. Webb for helpful discussions and to Miss S. Ryder and Mr L. J. King for valuable technical assistance.

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Studies on the Mode of Action of Excess of Vitamin A

3. RELEASE OF A BOUND PROTEASE BY THE ACTION OF VITAMIN A

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In the two preceding papers (Dingle, Lucy & Fell, 1961; Lucy, Dingle & Fell, 1961) it was shown that excess of vitamin A causes changes in both the composition and metabolism of cartilage cells and extensive breakdown of extracellular material. Hypo-osmotic treatment of embryonic cartilage released a proteolytic enzyme and degraded the matrix in a manner that was very similar to the effects of excess of vitamin A.

It was suggested that some of the effects of vitamin A might be explained if the vitamin causes the lysosomes to release a protease which would split the protein–polysaccharide complex of the matrix.

This paper presents evidence for a direct action of vitamin A upon isolated particulate components of liver cells and demonstrates some of the conditions under which the vitamin may release a bound protease.

METHODS

Tissue fractionations. Material was obtained from the livers of young adult rats. The tissue was cooled in ice-cold 0·25M-sucrose, weighed and a suspension (1 g. in 10 ml.) made, an all-glass motor-driven homogenizer being used. Debris and nuclei were removed by centrifuging at 600*g* for 5 min. in a Serval Superspeed centrifuge. The supernatant was either centrifuged at 10 000*g* for 25 min. to sediment all mitochondria-like particles, or else submitted to a fraction procedure similar to that of Appelmans, Wattiaux & de Duve (1955). For the latter process the extract was first spun at 10 000*g* for 5 min. to give the 'heavy' fraction, the pellet was washed twice under the same conditions and the very loosely packed material removed. This, together with the first supernatant, was spun at 10 000*g* for 25 min. to form the 'light' fraction.

All manipulations were done in a cold room at 4°, and, provided that care was taken when using the homogenizer, negligible hydrolytic activity was released from the particles.

Enzyme assay. The free protease was assayed in 0·1M-sodium acetate buffer, pH 4, with 2% (w/v) haemoglobin as substrate. The hydrolysis of the protein, which was estimated by the method of Anson (1938), was at 37° for 20 min. Activity was expressed as μg. of acid-soluble 'tyrosine' released. The reaction was stopped by the addition of ice-cold trichloroacetic acid and the mixture kept cold until the addition of the alkali (Gianetto & de Duve, 1955). Under these conditions the presence of sucrose did not appreciably interfere with the estimation of tyrosine. 'Total' activity was measured after suspending the particles in glass-redistilled water (10 ml./g. of original tissue) and storing at 4° for 30 min.

Treatment with vitamin A. A 1:5 suspension of the particles was made in 0·25M-sucrose and crystalline vitamin A alcohol (Roche Products Ltd.) dissolved in ethanol was added. An equal amount of ethanol was added to the control tubes, though at the maximum concentration of ethanol used (1%, v/v) there was no appreciable effect upon enzyme release. The particles were then incubated, usually at 37° for 45 min. under nitrogen, and at the end of the reaction period the suspensions were spun at 15 000*g* for 20 min. in the cold. The enzyme released was measured in the clear supernatant fluid and the amount expressed as a percentage of the total activity released by hypo-osmotic treatment.

RESULTS

Rate of release of the protease. The effect of incubation of a 'mitochondrial' suspension with increasing amounts of vitamin A has been investigated (Fig. 1). The protease activity of the controls

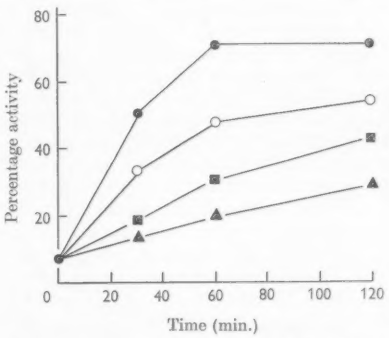

Fig. 1. Effect of vitamin A on the release of cathepsin from rat-liver lysosomes. 'Mitochondrial' fraction was suspended in 0·25M-sucrose and incubated at 37° under nitrogen, with varying concentrations of vitamin A. At the end of the incubation period the suspension was spun at 15 000*g* for 20 min. Proteolytic activity was measured in the supernatant and the amount is expressed as a percentage of the total activity released by hypo-osmotic treatment. ▲, Control; ■, 0·5 μg. of vitamin A/mg.; ○, 1·0 μg. of vitamin A/mg.; ●, 2·0 μg. of vitamin A/mg.

increased with time, but the vitamin caused a more rapid release of the enzyme. The experiment also demonstrates that the release of enzyme is related to the concentration of vitamin, and that the maximum rate of release is in the first hour of incubation.

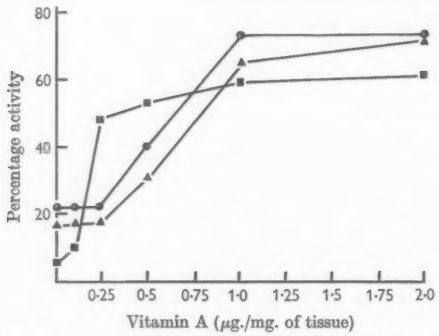

Fig. 2. Release of the protease from various mitochondrial fractions. Rat-liver mitochondria were fractionated in 0·25 M-sucrose as described in the text. The various fractions were incubated at 37° with vitamin A, for 45 min. under nitrogen. At the end of the incubation period the suspensions were spun at 15 000*g* for 20 min. and the proteolytic activity was estimated in the supernatant. ■, 'Light' mitochondrial fraction; ●, 'heavy' mitochondrial fraction; ▲, whole mitochondrial fraction.

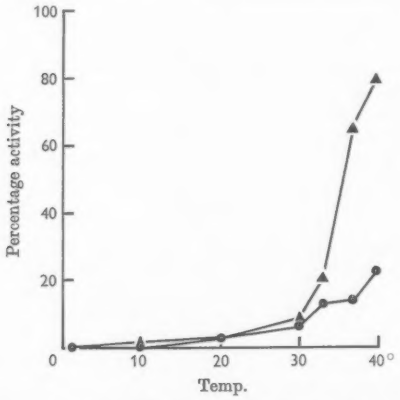

Fig. 3. Effect of temperature upon the release of cathepsin by vitamin A. Rat-liver mitochondria, suspended in 0·25 M-sucrose, were incubated for 45 min. under nitrogen with vitamin A (1 μg./mg. of tissue). At the end of the experimental period the suspensions were spun at 15 000*g* for 20 min. and proteolytic activity was measured in the supernatant. ▲, Vitamin A (1 μg./mg. of tissue); ●, control.

Release of the protease from various 'mitochondrial' fractions. The 'light' mitochondrial fraction that was obtained in this work appears to be more sensitive to the action of the vitamin than the 'heavy' or the whole fractions. 50 % of the total activity of the 'light' fraction is released by 0·25 μg. of vitamin A/mg. of original tissue after 45 min. at 37°, whereas three times this concentration is required to liberate 50 % of the activity of the whole fraction (Fig. 2). This difference may be due to higher ratios in the light fraction of lysosomal to non-lysosomal protein, or to lipoprotein, both of which may compete for the available vitamin.

Though all three preparations show an increasing release of protease with rising concentrations of vitamin A, the 'light' fraction exhibits a considerable release of enzyme over a narrow range of concentrations.

This rise in proteolytic activity under the influence of increasing amounts of the vitamin must be due to an increased liberation of the enzyme, since the vitamin has no effect upon the enzyme after it has been liberated under hypo-osmotic conditions.

Effect of temperature and pH *upon enzyme release.* The action of the vitamin upon the enzyme-containing particles is influenced by temperature (Fig. 3). Between 1° and 30° the vitamin has little or no effect, but above 30° there is an extremely rapid increase in the percentage activity released after incubation for 45 min. with 1 μg. of vitamin A/mg. of original tissue. At 40° there is also an increase in the thermal activation but this is small compared with the effect of the vitamin.

All the experiments so far reported have been performed by incubating the intracellular particles in 0·25 M-sucrose dissolved in glass-redistilled water of approximately neutral pH. Appelmans & de Duve (1955) reported that increase in acidity favoured the thermal activation of rat-liver lysosomes; it was therefore decided to compare the effects of different pH values on the thermal activation. The release of protease by the lysosomes was measured after incubation, with and without vitamin A, for 45 min. at 37° in 0·25 M-sucrose containing 0·02 M-sodium acetate buffers of differing pH values.

Below pH 5 (Fig. 4) there is a considerable increase in thermal activation and this is not appreciably altered by the presence of the vitamin. Above pH 5 the particles are stable in the absence of vitamin A, but the addition of the vitamin (1·0 μg./mg. of original tissue) is much more effective at neutral than at acid pH.

Effect of pH *upon the released enzyme.* Gianetto & de Duve (1955) reported that cathepsin released from rat-liver lysosomes by hypo-osmotic treat-

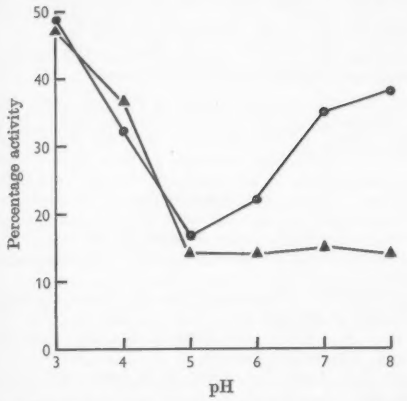

Fig. 4. Effect of pH upon the release of proteolytic activity from rat-liver lysosomes by vitamin A. 'Mitochondria' were suspended in 0·25 M-sucrose containing 0·02 M-sodium acetate buffer. The suspensions were incubated at 37° for 45 min. under nitrogen with 1 μg. of vitamin A/mg. of tissue. At the end of the incubation period the suspensions were spun at 15 000*g* for 20 min. and proteolytic activity was estimated in the supernatant. ●, Vitamin A (1 μg./mg. of tissue); ▲, control.

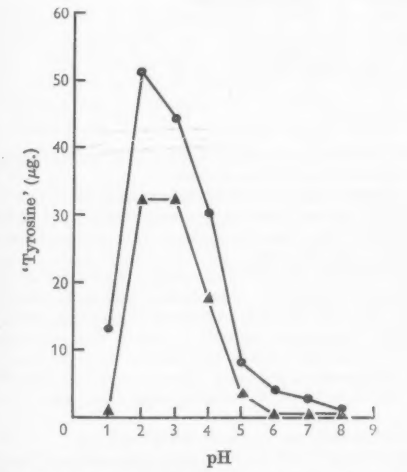

Fig. 5. Effect of pH upon the released enzyme. The cathepsin was released from rat-liver lysosomes by treatment with vitamin A (1 μg./mg. of tissue) and also by hypo-osmotic treatment. The enzyme solutions were incubated for 20 min. at 37° with 2% haemoglobin in 0·1 M-sodium acetate buffers of different pH values. The acid-soluble tyrosine released was estimated as indicated in the text. ●, Enzyme released by vitamin A; ▲, enzyme released by hypo-osmotic treatment.

ment was more active at pH 3·6 than at pH 5·0; this is confirmed by the results of the present experiments (Fig. 5), which also show that the enzyme released by vitamin A has the same pH optimum against haemoglobin as that liberated by hypo-osmotic treatment.

DISCUSSION

In the preceding paper (Dingle *et al.* 1961) it was shown that the effects of vitamin A on cartilaginous limb-bone rudiments could be simulated by hypo-osmotic treatment, and also that the hypo-osmotic effects were probably due to the action of an intracellular protease, which was able to break some part of the protein–polysaccharide complex of the matrix. In order to relate this model system, in which the enzyme is released by hypo-osmotic treatment, to the action of the vitamin on living tissues an important step was to find if the vitamin was able to liberate a protease by the direct addition of the chemically pure compound to the enzyme-containing particulate components of the cells.

The results presented in this paper show that a proteolytic enzyme, with an acid pH optimum, similar to the enzyme liberated from the cartilaginous rudiments by hypo-osmotic treatment, is rapidly released by treatment of the 'mitochondrial' fraction of rat liver with vitamin A. Rat liver was used as a source of material as Gianetto & de Duve (1955) have shown that stable cathepsin-containing particles, the lysosomes, can easily be obtained from this tissue. Preliminary observations upon homogenates of embryonic cartilage indicate that the vitamin probably has a similar effect on the particulate components of this tissue also. Since no evidence could be found that the activity of the protease liberated from rat-liver lysosomes by hypo-osmotic treatment is affected by the presence of the vitamin, it is probable that the increased proteolytic activity of the supernatant is due to release of the bound enzyme under the influence of vitamin A. There is no direct evidence at present to indicate the way in which the vitamin alters the permeability of the particles, though the dependence of the vitamin A effect upon temperature and pH might perhaps suggest an enzymic mechanism.

The above results may indicate the mechanism by which the vitamin, acting at a subcellular level, is able to alter so drastically the composition of the extracellular material. Mainly because the breakdown of matrix was initially of greatest interest and also because of the ease of assay, this paper has dealt only with the release of a proteolytic enzyme from the particulate preparations. de Duve (1959) has demonstrated the presence of a number of

hydrolases in the particles he terms lysosomes. Besides a cathepsin, these include a ribonuclease, a deoxyribonuclease, a phosphatase, a glucuronidase and a sulphatase. As described in the first paper of this series, the action of the vitamin upon the cartilaginous rudiments grown in culture causes a complex series of changes, including loss of ribonucleic acid, amino sugars and dry weight. It seems very likely that the vitamin may release some, or all, of these bound hydrolases and that this complex of enzymes may cause many of the changes seen in the tissue treated with vitamin A.

SUMMARY

1. Particulate preparations from rat liver suspended in 0·25 M-sucrose and treated with vitamin A release a proteolytic enzyme with an acid pH optimum.

2. The action of the vitamin on the particles is dependent upon temperature and pH.

3. The enzyme, after release by hypo-osmotic treatment, is not affected by the vitamin.

4. The relationship of this effect to the action of vitamin A on intact tissue is discussed.

The author is indebted to Dr H. B. Fell and Dr J. A. Lucy for helpful discussions and to Mr J. A. Thomson for technical assistance.

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Biochem. J. (1961) **79**, 512

A Method for the Chromatographic Detection of Penicillins and Related Compounds and of Penicillinase

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The intact penicillin molecule is not oxidized by molecular iodine, but after the β-lactam ring is opened—for example, when penicillin is inactivated by the enzyme penicillinase—the resulting penicilloic acid rapidly reacts with 8–9 equiv. of iodine (Alcino, 1946).

Iodine also reacts with many sulphur compounds related to penicillin and can be used to detect these on paper chromatograms. In combination with penicillinase it will detect those penicillins whose β-lactam ring is opened by the enzyme. Alternatively, the method can be used to detect penicillinase. The method has been developed in this Institute over the past few years.

EXPERIMENTAL

The reagents are made up as follows:

Starch–iodine solution: 0·01 M-iodine in 3 mM-potassium iodide, 10 ml.; M-phosphate buffer, pH 7·0 (54 g. of KH_2PO_4 and 84·6 g. of Na_2HPO_4 in 1 l. of water), 1 ml.; 2% (w/v) sodium starch glycollate (British Drug Houses Ltd.) in water (a crystal of thymol is added as preservative), 9 ml. The three stock solutions are stable, and the mixture is stable for several days at least.

Penicillin solution: sodium benzylpenicillin, 60 mg./ml. (100 000 i.u./ml.), freshly prepared, 1 ml.; M-phosphate buffer, pH 7·0, 1 ml.; water, 8 ml. This solution must be freshly made each day.

Penicillinase solution: Penicillinase solution of at least 1000 enzyme units/ml. A convenient method for preparing high-titre penicillinase from *Bacillus cereus* (strain 569/H or 5/B) has been given by Pollock (1957). This preparation usually contains 2000–10 000 enzyme units/ml., and is stable for some months at 4°: the enzymes from these two strains have given identical results when used in the methods described below.

Two methods are suggested for general use:

(1) Method A, for the detection of penicillin and related compounds. The paper is sprayed with starch–iodine solution and observed for 2 min. Most compounds, related to penicillin, containing potential sulphydryl groups immediately give pale spots on a deep-blue background, except for those with a closed β-lactam ring. The paper is now sprayed with a freshly made mixture of equal amounts of the starch–iodine and the penicillinase solutions. Additional pale spots will appear at the site of penicillins whose β-lactam ring is opened by the penicillinase. The

penicillinase solution will itself take up some iodine because of reducing-compounds in the medium, and should therefore be mixed as above with the starch–iodine solution before use. Generally a considerable amount of free iodine will remain in the mixture; if not, more starch–iodine solution should be added until the mixture remains dark blue for several minutes. The two penicillinases recommended above are so slowly inactivated by iodine under these conditions that the results are not affected.

(2) Method B, for the detection of penicillinase (i.e. penicillin β-lactamase). The paper is sprayed with a mixture of equal parts of the starch–iodine and penicillin solutions. The presence of penicillinase is demonstrated by the appearance of pale spots on the blue background and the rate of appearance of clear areas gives an indication of the amount of enzyme present. Any compounds which destroy iodine without enzyme treatment may be located by spraying first with starch–iodine solution, as in method A. To avoid a false negative result caused by inactivation of the enzyme by iodine the penicillin solution may be sprayed first, and followed after 10 min. by the starch–iodine solution. This immediately shows up all areas where the penicillin has been destroyed but the relative amounts of enzyme in different spots cannot now be estimated.

The blue colour on the paper will slowly fade as the iodine volatilizes, and the spots must therefore be marked. Some persons are hypersensitive to penicillin, and it is advisable to spray the penicillin solution in a suitable fume cupboard or hood.

RESULTS AND DISCUSSION

Table 1 shows the sensitivity of the method to compounds 'spotted' on Whatman no. 1 filter paper. Method A is about ten times as sensitive as the iodoplatinic acid reagents of Winegard, Toennies & Block (1948) for reduced-sulphur compounds, and has a sensitivity about equal to that of the iodine–azide spray (Chargaff, Levine & Green, 1948).

The following compounds were found to take up iodine immediately: benzylpenicilloic acid, benzylpenicilloic acid amide, D-penicillamine, thiazolidine-4-carboxylic acid, α-ethyl benzyloxypenicilloate. The following penicillins took up iodine rapidly only after spraying with *B. cereus* 569/H penicillinase: benzyl-, *p*-aminobenzyl-, penten-2-yl-, heptyl- and phenoxymethyl-penicillin and 6-(α-phenoxypropionamido)penicillanic acid; but 6-amino- and 6-(2:6-dimethoxybenzamido)-penicillanic acid did so only slowly. Cephalosporin C took up no appreciable amount of iodine even after spraying with the enzyme. *p*-Hydroxybenzylpenicillin takes up in the course of a few minutes approximately 4 equiv. of iodine (presumably due to iodination of the benzene ring) and can therefore slowly decolorize the reagent before it is split by penicillinase.

Negative results obtained with method A do not exclude the possible presence of a penicillin. Three cases may be considered where a negative result could be obtained: (i) where the penicillin is converted by the enzyme into a penicilloic acid, which does not react rapidly with iodine (however, no penicilloates derived from the thiazolidine nucleus of penicillin are yet known which do not react readily); (ii) where the penicillin has a low affinity for the enzyme and is therefore hydrolysed too slowly to give a positive response before the iodine evaporates completely from the paper; for example, 6-(2:6-dimethoxybenzamido)penicillanic acid (Celbenin) is hydrolysed only slowly by the penicillinase produced by *Staphylococcus aureus* 524 SC, for which it has a low affinity (R. Novick, personal communication); (iii) the penicillin may not be a substrate at all.

There is, however, no reason why very much larger amounts of purified penicillinase (Pollock, Torriani & Tridgell, 1956; Kogut, Pollock & Tridgell, 1956) cannot be used to increase the sensitivity for those compounds that are only slowly hydrolysed. The use of benzylpenicillin in method B seems to exclude false negative results, since all the penicillinases so far examined split benzylpenicillin very readily. The possibility of a

Table 1. *Sensitivity of the methods*

The figures in parentheses give the approximate amount of the substance per unit area when 'spotted' on Whatman no. 1 filter paper.

Substance	Amount readily detected within 2 min.	Amount just detected after 15–20 min.	Method used
Sodium benzylpenicillin	500 μg./ml. ($3\,\mu$g./cm.^{2})	100 μg./ml. ($0{\cdot}6\,\mu$g./cm.^{2})	A
6-Aminopenicillanic acid	10 mg./ml. ($60\,\mu$g./cm.^{2})	500 μg./ml. ($3\,\mu$g./cm.^{2})	A
Bacillus cereus 569/H or 5/B penicillinase (Pollock, 1957) or *Bacillus subtilis* 749 penicillinase (Kushner, 1960)	150 units/ml. (0·9 unit/cm.^{2})	1·5 units/ml. (0·009 unit/cm.^{2})	B
Staphylococcus aureus 524 SC penicillinase	200 units/ml. (1·2 units/cm.^{2})	2 units/ml. (0·012 unit/cm.^{2})	B

false positive result is also slight since so far no enzyme is known, other than that type of penicillinase (penicillin β-lactamase) for which the test was devised, which attacks penicillin to yield a product reacting with iodine.

SUMMARY

1. A method is described for the chromatographic detection of penicillins and related compounds and of penicillinase based on the reaction of iodine with penicilloic acids.

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Purification of Latent Phenolase from Broad-Bean (*Vicia faba*) Leaves

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The paucity of information on the nature and function of the plant phenolases is apparent from a recent review (Bonner, 1957) on this family of oxidases. An interesting contribution has been the large-scale fractionation and purification of mushroom phenolase by Kertesz & Zito (1957). More recently reports have appeared of the chromatography on cellulose ion-exchange agents of phenolase from mushroom (Frieden & Ottesen, 1959), *Polyporus versicolor* (Malmström, Fähraeus & Mosbach, 1958) and from mammalian tissue (Brown & Ward, 1958). A latent phenolase is present in the leaves of the broad bean, and Kenten (1957, 1958) has studied in some detail the activation of this enzyme in a partially purified preparation. During the course of work in these laboratories on the characterization of the proteins of the broad-bean leaf, various fractions, as judged by their colour, were obviously contaminated with Kenten's latent phenolase. This brief paper describes the chromatographic method used quite early in the work for the removal of the enzyme from pigments and from the major protein components of the leaf juice.

EXPERIMENTAL

Preparation of leaf extract. The broad-bean plants (Windsor Improved) were grown to various stages of maturity in a garden or in John Innes Compost in a heated greenhouse. The leaves (50–100 g. fresh weight) were vacuum-infiltrated with a solution containing 0·03M-potassium ascorbate, 0·2 mM-sodium ethylenediaminetetra-acetate and 0·03M-potassium phosphate buffer, pH 7·5. Excess of solution was then squeezed from the leaves before they were ground with the aid of a little sand in a pestle and mortar at 4°. The suspension was centrifuged at 20 000*g* for 45 min., and the supernatant dialysed against many changes of buffer containing 5 mM-ascorbate, 4 mM-phosphate and 4 mM-citrate, adjusted to pH 6·5 with potassium hydroxide. The latent phenolase solution was freeze-dried, and the residue was taken up in 2–3 ml. of water, centrifuged if necessary to clarify, and dialysed for 1 hr. against 5 mM-potassium phosphate buffer at pH 7.

Column chromatography. Diethylaminoethylcellulose (DEAE-cellulose) was prepared from Solka Floc SW-40B (Johnsen, Jorgensen and Wettre Ltd., London, E.C. 4) according to Peterson & Sober (1956) but with only half the quantity of the reagent α-chlorotriethylamine hydrochloride. The resulting adsorbent contained 0·50 m-equiv. of basic nitrogen/g. A column of DEAE-cellulose (30 cm. × 1 cm.) was prepared from a slurry in 5 mM-potassium phosphate buffer, pH 7, and packed down under a pressure of 8 lb./in.². The phenolase solution was transferred to the column and eluted rapidly with 5 mM-potassium phosphate buffer at pH 7. After 20 ml. had passed through the column, 0·5M-potassium hydroxide was applied through a 100 ml. mixing chamber to establish a concentration gradient and a pH gradient to 8·5. Duplicate samples of 0·1 ml. were taken from 2 ml. fractions for analysis by the Folin–Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) for protein and by β-(3:4-dihydroxyphenyl)-DL-alanine–Teepol for phenolase. The latter reagent was prepared from 5 ml. of Teepol (Anionic detergent, Shell Chemical Co.), 30 mg. of β-(3:4-dihydroxyphenyl)-DL-alanine and 0·05M-potassium phosphate at pH 6 to a volume of 50 ml. This reagent (0·5 ml.) and 4 ml. of buffer, pH 6, were added to each portion, and the extinction of the

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orange-coloured solution was read at 495 mμ after a period of 15–20 min. at room temperature. Although this method is not as accurate as measuring the rate of change of extinction for each enzyme concentration, it is quite adequate for monitoring the column effluent. A second column of DEAE-cellulose was operated at pH 7 with increasing ionic strength only.

Catecholase activity. Catecholase activity was estimated by the method described by Dawson & Magee (1955) which involves measuring the production of *o*-benzoquinone as a function of time from a given amount of enzyme. A 'unit of activity' corresponds to *o*-benzoquinone production of $1{\cdot}49 \times 10^{-8}$ mole/sec.

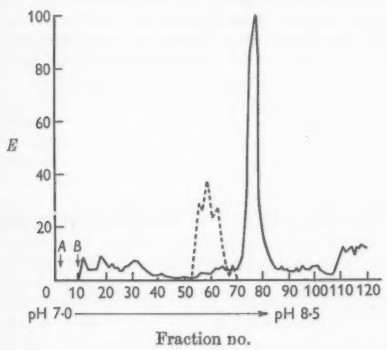

Fig. 1. Chromatography of whole juice from 50 g. of fresh leaves on a column (30 cm. × 1 cm.) of DEAE-cellulose; 2 ml. fractions. —, Folin–Lowry method with 0·1 ml. portions, extinction measured at 570 mμ; - - - -, phenolase activity of 0·1 ml. portions in terms of coloured oxidation products formed in 15 min. from β-3:4-dihydroxyphenylalanine in the presence of Teepol, extinction measured at 495 mμ. *A*, 5 mM-Phosphate buffer at pH 7·0; *B*, 0·5M-KOH through a 100 ml. mixing chamber to produce gradient to pH 8·5.

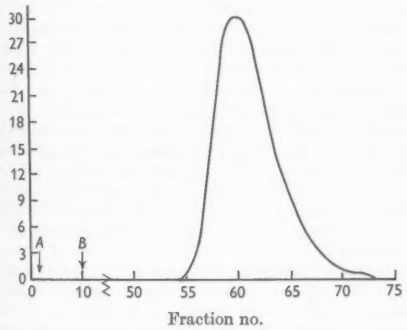

Fig. 2. Rechromatography of latent phenolase on a column (45 cm. × 1 cm.) of DEAE-cellulose. Analysis of 2 ml. fractions was as in Fig. 1. *A*, 5 mM-Phosphate buffer at pH 7·0; *B*, gradient change to 0·5M-phosphate at pH 7·0 through a 100 ml. mixing chamber. Specific activity of pooled peak was 2000–3000 units/mg. of nitrogen.

RESULTS

A chromatogram of whole juice from broad-bean leaves on DEAE-cellulose is shown in Fig. 1. The bulk protein of the extract can be divided into a number of fractions completely free of phenolase activity, and although protein is eluted continuously only a small amount is associated with the enzyme. Most of the total protein is eluted before pH 8·5 is reached; increasing the ionic strength of the eluent without increasing the pH causes considerable overlap of the main protein peak with the phenolase activity. The pooled latent phenolase fractions were concentrated and the sample rechromatographed on another column of DEAE-cellulose. A single and fairly symmetrical peak was obtained as shown in Fig. 2. The overall yield after passage through the two columns was greater than 90 %. The chronometric method of Dawson & Magee (1955) seemed the most convenient way for assessing the specific activity of the enzyme with catechol as substrate. According to this method juice from broad-bean leaves had a specific activity, after activation with Teepol, of 4–5 units/mg. of nitrogen. Preparations obtained by chromatography on the first and second DEAE-cellulose columns had specific activities in the range 1800–2500 and 2000–3000 units/mg. of nitrogen respectively.

Unlike all active phenolases so far reported in the literature, activated broad-bean phenolase is strongly adsorbed on DEAE-cellulose, the semi-translucent appearance of the adsorbent changing during the process of adsorption to an opaque white. No chemical modification of the adsorbent appears to take place, the nitrogen content and titration curve remaining unchanged.

No further purification of the enzyme has been attempted beyond the above, but in view of its stability and the high yields obtainable it would seem to be an ideal member of the plant phenolases for structural investigation and for further metabolic studies.

SUMMARY

1. Latent phenolase from broad-bean leaves may be removed from pigments and the major protein components of the leaf juice in one operation by gradient elution from a column of diethylaminoethylcellulose.

2. Rechromatography of the enzyme on the same adsorbent yields a preparation of high catecholase activity in 90 % overall yield.

3. Unlike other known active phenolases the enzyme released from a broad-bean leaf extract by Teepol is adsorbed strongly on diethylaminoethylcellulose.

It is a pleasure to acknowledge the technical assistance of Elizabeth P. McAllan.

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An Enzyme from Rat Liver Catalysing Conjugations with Glutathione

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Many compounds are excreted in urine and bile as mercapturic acids, which are thio ethers of acetylcysteine, and there has been considerable speculation on the source of the cysteine used for the biosynthesis of these compounds. Evidence that glutathione is involved was supplied by the work of Barnes, James & Wood (1959), who found that the levels of glutathione in the liver decreased when mercapturic acid-forming compounds were administered to rats. Furthermore, when *p*-chlorobenzyl chloride was given to a rabbit, investigations with paper chromatography provided evidence for the presence of *S*-(*p*-chlorobenzyl)-glutathione in the liver, and this compound could be converted into *S*-(*p*-chlorobenzyl)cysteine by liver slices and homogenates and by glutathionase (Bray, Franklin & James, 1959*a*). The *N*-acetylation of some of the *S*-substituted cysteines to the corresponding mercapturic acids was demonstrated *in vivo* and in tissue preparations *in vitro* (Bray, Franklin & James, 1959*b*).

It has been shown that rat-liver slices convert naphthalene into *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (Booth, Boyland & Sims, 1960*b*) and 1:2-dihydronaphthalene into *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)glutathione. This latter reaction could also be accomplished by rat-liver microsomes in the presence of reduced triphosphopyridine nucleotide, oxygen and glutathione, and the amount of glutathione derivative formed was increased by the addition of the soluble fraction of rat liver (Booth, Boyland, Sato & Sims, 1960*a*).

This paper describes the partial purification and some of the properties of the liver enzyme which catalysed the formation of glutathione derivatives from various compounds.

EXPERIMENTAL

Materials. Triphosphopyridine nucleotide (TPN) as the dihydrate of the sodium salt, reduced TPN ($TPNH_2$) as the sodium salt, diphosphopyridine nucleotide (DPN), reduced DPN ($DPNH_2$) as the disodium salt, glucose 6-phosphate as the disodium salt, flavin mononucleotide as the monosodium salt dihydrate, adenosine 5-triphosphate (ATP) as the disodium salt and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co. Pyrophosphate buffers were prepared from $K_4P_2O_7$ and $Na_2H_2P_2O_7$ and phosphate buffer from KH_2PO_4 and Na_2HPO_4. Concentrations of the constituents of the reaction mixtures refer to the final concentrations at the beginning of the incubations.

S-(2-*Chloro*-4-*nitrophenyl*)-L-*cysteine.* This was prepared from 2-chloro-4-nitroaniline by the method of Parke & Williams (1951), when it formed pale-yellow needles from aq. ethanol, m.p. 176–178° (decomp.) (Found: N, 10·15; Cl, 12·45. Calc. for $C_9H_9O_4N_2ClS$: N, 10·1; Cl, 12·8%). Bray *et al.* (1959*b*) report m.p. 182° for this compound prepared by the method of Suter (1895). Acetylation of the compound with acetic anhydride in 2N-NaOH yielded *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine in cream needles from ethanol, m.p. 193°. Bray, James & Thorpe (1957) report m.p. 192–194° for this derivative.

S-(2-Chloro-4-nitrophenyl)glutathione. This compound was prepared from glutathione (2 g.) and 2-chloro-4-nitroaniline (1·1 g.) by the method described for the preparation of 1-naphthylglutathione (Booth *et al.* 1960*b*). *S-(2-Chloro-4-nitrophenyl)glutathione* (0·95 g.) separated from aq. 90% ethanol–ether as a pale-yellow powder, m.p. 214° (decomp.) (Found: N, 12·6; Cl, 7·8; S, 6·8. $C_{16}H_{19}O_8N_4ClS$ requires N, 12·1; Cl, 7·7; S, 6·9%). Like the corresponding cysteine derivative, the compound turned brown on exposure to daylight. When the compound was heated with HBr (sp.gr. 1·7) for 4 hr. and the products were examined on paper chromatograms (with solvent 1, Table 2), compounds indistinguishable from *S*-(2-chloro-4-nitrophenyl)-cysteine, glycine and glutamic acid were detected.

N-Acetyl-L-cysteine was prepared by the method of Pirie & Hele (1933) and *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and *trans*-9:10-dihydro-9:10-dihydroxyphenanthrene by reduction of the corresponding *o*-quinones with lithium aluminium hydride (Booth, Boyland & Turner, 1950). *trans*-1:2-Dihydro-1:2-dihydroxyphenanthrene was isolated from the urine of a rabbit dosed with phenanthrene (Boyland & Wolf, 1950).

A solution of oxidized glutathione (GSSG) was prepared by adding a small excess of 50 mM-potassium ferricyanide to a solution of 50 mM-GSH (10 ml.) and keeping the mixture at room temperature for 1 hr. The solution was adjusted to pH 4 with acetic acid and the GSSG absorbed on charcoal (400 mg.). The charcoal was filtered off and washed with water until the washings were free of potassium ferricyanide (no blue colour with $FeCl_2$). The GSSG was eluted with 10 ml. of methanol–benzene–aq. NH_3 (sp.gr. 0·88) (87:10:3, by vol.). The eluate was evaporated to dryness and the GSSG was dissolved in 0·1 M-pyrophosphate buffer, pH 8·0 (10 ml.).

Tissue preparations. The liver fraction soluble in phosphate buffer was prepared by homogenizing livers from male adult rats in 4 vol. of 0·1 M-phosphate buffer, pH 7·4, with a Potter & Elvehjem (1936) type of homogenizer with a Teflon pestle. The homogenate was centrifuged for 30 min. at 2000*g* and the supernatant centrifuged for 1 hr. at 95 000*g* (centre of tube) in a Spinco model L preparative ultracentrifuge with rotor no. 40. The supernatant from the high-speed centrifuge was dialysed against water and the precipitate which separated was removed by centrifuging for 30 min. at 2000*g*. The supernatant was adjusted to pH 7·4 with 0·1 M-$K_4P_2O_7$ and stored at −2°. This preparation is referred to as the soluble liver fraction. Kidneys from male adult rats were homogenized in 4 vol. of 0·154 M-NaCl immediately before use. All operations were carried out at between 3° and 8°.

Metabolism of 3:4-dichloronitrobenzene. This was investigated by incubating the following reaction mixtures at 37° for 1 hr. (1) Soluble liver fraction (0·5 ml.), GSH, cysteine or acetylcysteine (5 mM) and 3:4-dichloronitrobenzene (1 mM, added in 0·1 ml. of ethanol) in a total volume of 6 ml. of 0·1 M-pyrophosphate buffer, pH 8·0; (2) *S*-(2-chloro-4-nitrophenyl)glutathione (1 mM) and kidney homogenate (1 ml.) in a total volume of 6 ml. of 0·1 M-pyrophosphate buffer, pH 8·0; (3) *S*-(2-chloro-4-nitrophenyl)-cysteine (1 mM) and slices of liver (approx. 5 g. wet wt.) from adult rats in 50 ml. of Ringer phosphate solution, pH 7·4 (Umbreit, Burris & Stauffer, 1949). At the end of the incubation period the reaction mixtures were studied by paper chromatography.

Paper chromatography. The reaction mixtures were added to acetic acid (0·5 ml.) and centrifuged, and the supernatant was added to activated charcoal (50 mg.), shaken and again centrifuged. This supernatant was discarded and the charcoal was resuspended in water (6 ml.) and filtered. The products were eluted from the charcoal with 10 ml. of methanol–benzene–aq. NH_3 (sp.gr. 0·88) (87:10:3, by vol.) and the eluate was evaporated to a small volume and applied to Whatman no. 1 paper for chromatography. Descending chromatograms were developed for 18 hr. with one of the solvent systems listed in Table 2. The dried chromatograms were examined under u.v. light and then sprayed with (1) 20% Na_2CO_3 (Bray *et al.* 1957) or (2) 0·1 M-$K_2Cr_2O_7$–acetic acid (1:1), followed by 0·1 M-$AgNO_3$ (Knight & Young, 1958), or (3) diazotized *p*-nitroaniline (0·02% in 0·1 N-HCl), followed by aq. 10% (w/v) Na_2CO_3, or (4) diazotized *p*-nitroaniline (0·02% in 4 N-HCl), after which the papers were heated at 70° until colours appeared (about 5 min.) and then sprayed with aq. 10% (w/v) Na_2CO_3. Other papers were dipped in a solution of ninhydrin (0·4%) in acetone, heated in an oven at 70° for 5 min. and then dipped in a solution of $Cu(NO_3)_2$ (0·2%) in acetone.

Estimation of enzyme activity. The substrate chosen for the estimation of enzyme activity was 3:4-dichloronitrobenzene, because of the change in the ultraviolet-absorption spectrum which occurs when this compound is converted into *S*-(2-chloro-4-nitrophenyl)glutathione (Figs. 1 and 2). In obtaining the readings in Fig. 2, a cell containing all the constituents of the reaction mixture except 3:4-dichloronitrobenzene was used as the blank, so that the rate of increase of E at 344 mμ is a measure of the rate of formation of *S*-(2-chloro-4-nitrophenyl)glutathione. This increase was measured on a Unicam SP. 500 spectrophotometer with a Unicam SP. 570 constant-temperature-cell housing. The reaction mixtures contained GSH (5 mM), various concentrations of 3:4-dichloronitrobenzene (added in 0·1 ml. of ethanol) and enzyme in a total volume of 3·0 ml. of 0·1 M-pyrophosphate buffer, pH 8·0. The enzyme was diluted so that the rate of increase in E was less than 0·2/min. The reactions were carried out at 37°, the constituents being brought to this temperature before the determination and the reaction was started by the addition of the enzyme solution to the cell. After stirring, readings at 344 mμ were taken at 30 sec. intervals for 5·5 min., beginning 30 sec. after mixing. Under these conditions the initial reaction rates were constant for at least 10 min. and 1 unit of enzyme is defined as that amount which will form 1 μmole of *S*-(2-chloro-4-nitrophenyl)glutathione/min. Specific activity is expressed as units/mg. of protein. Protein was estimated by a modification of the biuret method (Fincham, 1954) or by the method of Warburg & Christian (1941).

Location of the enzyme. To determine the intracellular location of the enzyme, a 1 in 10 homogenate of adult male rat liver in 0·25 M-sucrose was prepared as described for the phosphate-buffer homogenate. The sucrose homogenate was fractionated by the method of Schneider & Hogeboom (1950), except that the microsome fraction was separated from the soluble fraction by centrifuging at 95 000*g* (centre of the tube) for 1 hr. in a Spinco model L preparative ultracentrifuge with rotor no. 40. The particulate fractions were suspended in 0·1 M-pyrophosphate buffer, pH 8·0, in volume equivalent to those of the original homogenate and the fractions were diluted with this buffer for measurements of enzyme activity.

To determine the distribution of the enzyme in the soluble fraction of various rat tissues, the tissues were homogenized in 0·1 M-phosphate buffer, pH 7·4, and the phosphate buffer-soluble fractions prepared as described for liver. Blood was added to an equal volume of water and after 10 min. an equal volume of 0·1 M-phosphate buffer was added. The supernatant after centrifuging for 1 hr. at 95 000*g* was then used for measurements of enzyme activity.

Partial purification of the enzyme. Steps 1 and 2 are the homogenization of rat liver and the preparation of the soluble fraction as described above. *Step* 3. The undialysed soluble liver fraction was adjusted to pH 5·2 with acetic acid (approx. 0·55 ml./100 ml.), centrifuged for 15 min. at 2000*g* and the ppt. discarded. The supernatant was dialysed against water for 18 hr., centrifuged as before and the ppt. again discarded. The supernatant was adjusted to pH 8·0 with 0·1 M-$K_4P_2O_7$ and the centrifuging repeated. *Step* 4. To the supernatant from step 3, $(NH_4)_2SO_4$ (47·2 g./100 ml.) was added and, after 30 min., the ppt. was removed by centrifuging and discarded. A small portion of the supernatant was dialysed against water and used for activity measurements. *Step* 5. The supernatant from step 4 was adjusted to pH 4·5 with 5% acetic acid and centrifuged for 30 min. at 2000*g*. The ppt., which contained the activity, was dissolved in 0·1 vol. of 0·1 M-pyrophosphate buffer, pH 7·4, and stored in 0·5 ml. portions at −15°. Immediately before use a portion was allowed to thaw at room temperature and was diluted 40 times with 0·1 M-pyrophosphate buffer, pH 8·0, so that when 0·25 ml. of this solution was present in 3 ml. of reaction mixture the total dilution factor was 480 and the protein concentration was 0·0117 mg./ml. This gave a change in E at 344 mμ of 0·15 to 0·20/min. under the optimum conditions. All operations were carried out at between 3° and 8°. The partial purification of the enzyme by these steps is summarized in Table 1.

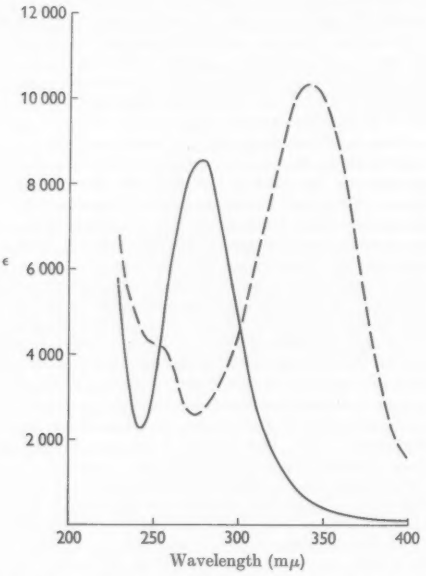

Fig. 1. Absorption spectra in 0·1 M-pyrophosphate buffer, pH 8·0: —, 3:4-dichloronitrobenzene; - - -, *S*-(2-chloro-4-nitrophenyl)glutathione.

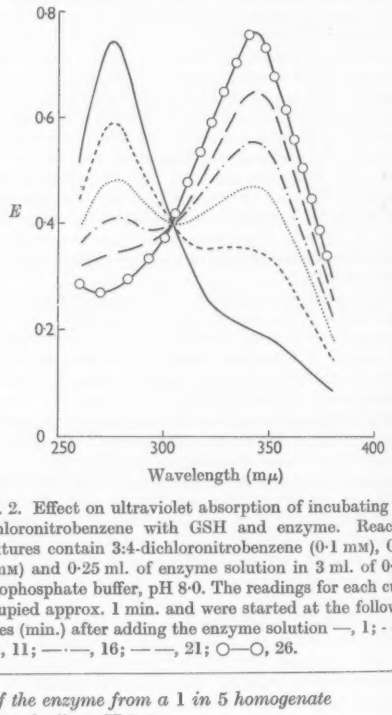

Fig. 2. Effect on ultraviolet absorption of incubating 3:4-dichloronitrobenzene with GSH and enzyme. Reaction mixtures contain 3:4-dichloronitrobenzene (0·1 mM), GSH (5 mM) and 0·25 ml. of enzyme solution in 3 ml. of 0·1 M-pyrophosphate buffer, pH 8·0. The readings for each curve occupied approx. 1 min. and were started at the following times (min.) after adding the enzyme solution —, 1; - -, 6; · · ·, 11; —·—, 16; — —, 21; ○—○, 26.

Table 1. *Summary of the partial purification of the enzyme from a* 1 *in* 5 *homogenate of rat liver in* 0·1 M-*phosphate buffer*, pH 7·4

Step	Volume (ml.)	Activity (units/ml.)	Total units	Protein (mg./ml.)	$10^2 \times$ Specific activity (units/mg. of protein)	Recovery (%)
(1) Whole homogenate	360	21	7560	45·1	47	100
(2) Soluble liver fraction	245	27	6615	26·2	104	87
(3) Precipitation at pH 5·2 and dialysis	270	19	5130	12·1	158	68
(4) $(NH_4)_2SO_4$ fractionation at pH 8·0	330	9	2970	1·4	643	39
(5) $(NH_4)_2SO_4$ fractionation at pH 4·5	33	90	2970	5·6	1607	39

Table 2. *Paper chromatography of* 3:4-*dichloronitrobenzene metabolites*

Solvent systems: (1) butanol–acetic acid–water (12:3:5); (2) butanol–acetic acid–water (2:1:1). All metabolites showed dark absorption in u.v. light and gave a positive reaction with the $K_2Cr_2O_7$–$AgNO_3$ reagent.

Reaction mixture or reference compound	R_F Solvent (1)	R_F Solvent (2)	Colour with ninhydrin and $Cu(NO_3)_2$	Colour with Na_2CO_3
3:4-Dichloronitrobenzene, dialysed soluble liver fraction and GSH	0·45	0·62	Red	Yellow
S-(2-Chloro-4-nitrophenyl)glutathione	0·44	0·61	Red	Yellow
S-(2-Chloro-4-nitrophenyl)glutathione and kidney homogenate	0·57	0·67	Red	Yellow
S-(2-Chloro-4-nitrophenyl)-L-cysteine	0·59	0·67	Red	Yellow
S-(2-Chloro-4-nitrophenyl)-L-cysteine and liver slices	0·89	0·86	Orange	Pale yellow
N-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	0·89	0·86	Orange	Pale yellow

Identification of glutathione conjugates with various substrates. Reaction mixtures contained soluble liver fraction (2 ml.), GSH (5 mM) and 0·1 M-pyrophosphate buffer, pH 8·0, in a final volume of 6 ml. The substrates were added in ethanol (0·2 ml.) to give a final concn. of 1 mM; some of the compounds were partially precipitated and with these the excess of substrate was allowed to remain in suspension. With the substrates iodobenzene, naphthalene, 1-chloronaphthalene and phenanthrene the following cofactors were also added: $DPNH_2$ (0·2 mM), nicotinamide (2 mM), TPN (0·2 mM), glucose 6-phosphate (2 mM), glucose 6-phosphate dehydrogenase (0·01%) and 1 ml. of microsome suspension prepared as described by Booth *et al.* (1960*a*). Two incubation flasks were used for each substrate, one containing 2 ml. of soluble liver fraction and the other either 2 ml. of soluble liver fraction which had been heated at 100° for 5 min. before the incubation or 2 ml. of buffer solution.

The mixtures were incubated at 37° for times which varied between 2 min. and 1 hr., according to the substrate, the same time being used for each pair of flasks containing the same substrate. The products were adsorbed on charcoal and eluted as described above, and the eluates were evaporated to dryness and methanol (0·25 ml.) containing aq. 1% NH_3 (sp.gr. 0·88) was added to the residues. For each substrate the same volume of the methanol extract from the two experiments (boiled and fresh soluble liver fraction) was applied with micropipettes to chromatography paper for comparison. With some substrates only a small fraction of the methanol was used, to avoid overloading. The dried chromatograms were treated with the ninhydrin reagent followed by the $Cu(NO_3)_2$ solution. The spots were cut out, the coloured compounds eluted with 50% methanol and the extinctions measured at 510 mμ on the Unicam SP. 500 spectrophotometer. A reaction mixture which contained soluble liver fraction and GSH but no substrate was treated in the same way for use as a blank.

In the experiments with naphthalene and phenanthrene, other paper chromatograms were treated with the diazotized *p*-nitroaniline reagents (3) and (4).

RESULTS

Metabolism of 3:4-*dichloronitrobenzene.* The conversion of 3:4-dichloronitrobenzene into *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine (Bray *et al.* 1957) in three stages is demonstrated by the results in Table 2. The first reaction, which is catalysed by the soluble liver fraction, is the replacement of the *para* chlorine atom by GSH to form *S*-(2-chloro-4-nitrophenyl)glutathione. If GSH is replaced by cysteine or acetylcysteine in the reaction mixture no corresponding derivatives can be detected on the chromatograms. Kidney homogenate removes glycine and glutamic acid from *S*-(2-chloro-4-nitrophenyl)glutathione to form *S*-(2-chloro-4-nitrophenyl)-L-cysteine, which is then acetylated by liver slices to the mercapturic acid, *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine.

Table 3. *Intracellular location of the enzyme*

Samples of the fractions of 1 in 10 rat-liver homogenate in 0·25 M-sucrose were incubated with GSH (5 mM) and 3:4-dichloronitrobenzene (1 mM) in 0·1 M-pyrophosphate buffer, pH 8·0 (total vol. 3 ml.).

Intracellular fraction	Activity (units/ml.)	Activity (as % of whole homogenate)
Whole homogenate	15·8	100
Nuclei	0·8	5
Mitochondria	0·8	5
Microsomes	0·7	4
Soluble fraction	13·0	82

Table 4. *Distribution of the enzyme in the soluble fractions of rat tissues*

Reaction mixtures contained GSH (5 mM), 3:4-dichloronitrobenzene (1 mM) and soluble liver fractions in 0·1 M-pyrophosphate buffer, pH 8·0 (total vol. 3 ml.).

Tissue	$10^2 \times$ Specific activity (units/mg. of protein)
Liver	96·2
Heart	2·9
Kidney	2·4
Lung	1·6
Spleen	0·5
Blood	0·1

Location of the enzyme. The estimations of enzyme activity in the intracellular fractions of a rat-liver homogenate in 0·25 M-sucrose shows that 82% of the activity is present in the soluble fraction (Table 3). The activities of the soluble fractions of various rat tissues are listed in Table 4, which shows that liver has between 30 and 200 times the activity of the other tissues investigated.

Some properties of the partially purified enzyme. The activity is completely destroyed by heating at 100° for 5 min. and is not affected by dialysis. The initial reaction rate under the conditions described is constant for at least 10 min. and is directly proportional to enzyme concentration when this is adjusted to give a change in E at 344 mμ of 0·2/min. or less. The pH–activity curve (Fig. 3) shows a broad region of optimum activity between 7·5 and 9·0, but a rapid decrease on either side of these values. Replacement of pyrophosphate buffer by Na_2CO_3–$NaHCO_3$ buffer, of the same molarity and pH, at the pH of optimum activity had no effect on the reaction rate.

GSH was used throughout at a concentration of 5 mM, and increasing this value produced no increase in enzyme activity. Although it could be demonstrated that a decrease in GSH concentration caused a decrease in activity, a quantitative study of this factor was not undertaken as it was uncertain what proportion of GSH remained in the reduced form during the experiments. No activity was observed when GSH was replaced by the same concentration of GSSG, cysteine or acetylcysteine. The absorption spectra of the GSH, cysteine and acetylcysteine derivatives of 3:4-dichloronitrobenzene are all very similar (cf. Bray *et al.* 1959*b*), so that if any reaction had taken place a change in E at 344 mμ would have been apparent. This confirms the results obtained with paper chromatography, in which GSH was the only one of these three SH compounds which was converted into a derivative of 3:4-dichloronitrobenzene by the soluble liver fraction.

Investigations on the effect of 3:4-dichloronitrobenzene concentration were restricted by the solubility (1 mM) of this substrate, and it was not possible to saturate the enzyme. However, the effect of various substrate concentrations below this value are shown in Fig. 4 in the form of a Lineweaver & Burk (1934) plot, and the K_m calculated from these values was 2·5 mM ($2{\cdot}5 \times 10^{-3}$ M). The inhibition of *S*-(2-chloro-4-nitrophenyl)glutathione formation by other compounds which were

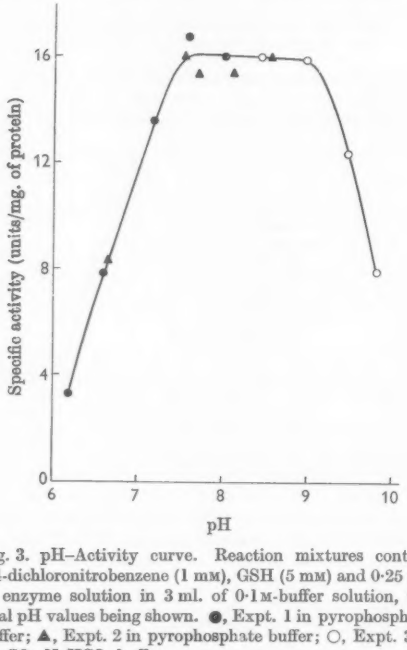

Fig. 3. pH–Activity curve. Reaction mixtures contain 3:4-dichloronitrobenzene (1 mM), GSH (5 mM) and 0·25 ml. of enzyme solution in 3 ml. of 0·1 M-buffer solution, the final pH values being shown. ●, Expt. 1 in pyrophosphate buffer; ▲, Expt. 2 in pyrophosphate buffer; ○, Expt. 3 in Na_2CO_3–$NaHCO_3$ buffer.

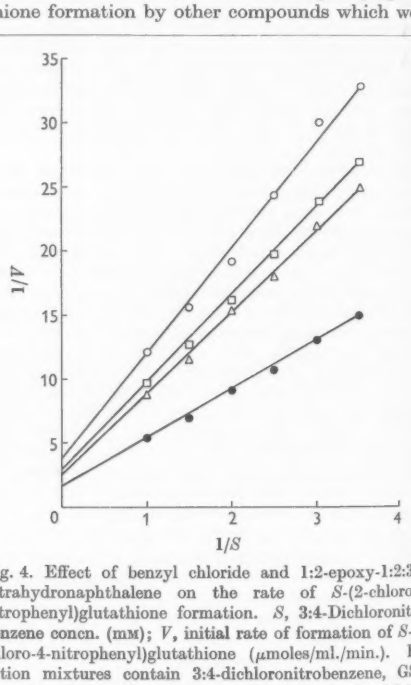

Fig. 4. Effect of benzyl chloride and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene on the rate of *S*-(2-chloro-4-nitrophenyl)glutathione formation. *S*, 3:4-Dichloronitrobenzene concn. (mM); *V*, initial rate of formation of *S*-(2-chloro-4-nitrophenyl)glutathione (μmoles/ml./min.). Reaction mixtures contain 3:4-dichloronitrobenzene, GSH (5 mM) and 0·25 ml. of enzyme solution in 3 ml. of 0·1 M-pyrophosphate buffer, pH 8·0, with the following additions: ●, none; △, 0·3 mM- and ○ 0·6 mM-1:2-epoxy-1:2:3:4-tetrahydronaphthalene; □, 0·6 mM-benzyl chloride.

shown by paper chromatography to form GSH derivatives is also shown in Fig. 4. At all the 3:4-dichloronitrobenzene concentrations studied, i.e. 1, 0·67, 0·5, 0·4, 0·33 and 0·285 mM, the addition of 0·6 and 0·3 mM-1:2-epoxy-1:2:3:4-tetrahydronaphthalene caused 55 and 40 % inhibition respectively, whereas at the same concentrations of 3:4-dichloronitrobenzene, 0·6 mM-benzyl chloride caused 44 % inhibition. Furthermore, when the concentration of 3:4-dichloronitrobenzene was kept constant at 1 mM, the addition of 0·2, 0·4, 0·6, 0·8 and 1 mM-benzyl chloride produced 17, 33, 43, 58 and 68 % inhibition respectively. Therefore, regarding 3:4-dichloronitrobenzene as the substrate and the other two compounds as inhibitors, the inhibition is of a non-competitive nature since it is independent of substrate concentration but dependent on inhibitor concentration.

The addition of the following compounds at a final concentration of 1 mM had no effect on the initial reaction rate: $TPNH_2$, $DPNH_2$, TPN, DPN, flavin mononucleotide, ATP and $MgSO_4$, ascorbic acid, cysteine, acetylcysteine, ethylenediaminetetra-acetate, KCN, NaF, $ZnSO_4$ and $MgCl_2$.

Formation of glutathione conjugates with various substrates. The formation of the GSH conjugates was demonstrated by the appearance of ninhydrin-positive spots when the substrates, GSH and soluble liver fraction were incubated in 0·1 M-pyrophosphate buffer, pH 8·0. With some substrates a GSH conjugate could be detected only when fresh soluble liver fraction was present in the incubation mixture, indicating that these reactions had been brought about by an enzyme in the liver fraction. Other substrates formed a GSH conjugate in the presence of boiled liver fraction, but a greater amount of the same derivative, as determined by the difference in extinction at 510 mμ after reaction with ninhydrin and $Cu(NO_3)_2$, when the liver fraction was fresh. It was assumed that in these cases both chemical and enzymic reactions had occurred. In no case, however, was the new ninhydrin-positive compound formed if either the substrate or GSH was omitted. The difference in extinctions observed between the two experiments with the same substrate ranged from a three- to a ten-fold increase. However, they could serve only to indicate that an enzymic as well as a chemical reaction had taken place, since a kinetic study with each substrate and the synthesis or isolation of the corresponding GSH derivative would be needed to put them on a quantitative basis. Benzyl chloride, which reacts chemically and enzymically, was used to confirm the specificity of the system for GSH. Paper chromatograms of reaction mixtures in which GSH had been replaced by GSSG showed that no reaction had taken place, and when cysteine or *N*-acetylcysteine replaced the GSH the chemical reaction only was observed. With the substrates iodobenzene, naphthalene, 1-chloronaphthalene and phenanthrene there was no formation of GSH conjugates unless microsomes and $TPNH_2$ were also added. The properties on paper chromatograms of the GSH conjugates detected in these reactions are listed in Table 5.

Naphthalene also yielded a compound which gave a blue colour with reagent 4 and was indistinguishable from *trans*-1:2-dihydro-1:2-dihydr-

Table 5. *Paper chromatography of glutathione conjugates*

Solvent systems are as described in Table 1. All conjugates gave pink colours with the ninhydrin–$Cu(NO_3)_2$ reagent. Type of reaction is indicated by A, chemical and B, enzymic.

Substrate	R_F of GSH derivative: Solvent (1)	R_F of GSH derivative: Solvent (2)	Type of reaction observed	Reference to mercapturic acid excretion in urine
3:4-Dichloronitrobenzene	0·45	0·62	B	Bray, James & Thorpe (1957)
2:4-Dichloronitrobenzene	0·42	0·60	B	Bray *et al.* (1957)
2:3:5:6-Tetrachloronitrobenzene	0·62	0·72	B	Bray, Hybs, James & Thorpe (1953)
3:4:5:6-Tetrachloro-1:2-epoxy*cyclo*hexane	0·42	0·64	B	—
Benzyl chloride	0·50	0·67	A, B	Bray, James & Thorpe (1958)
Bromoethane	0·33	0·52	A, B	Thomson, Maw & Young (1958)
1-Bromopropane	0·44	0·62	A, B	Grenby & Young (1959, 1960)
Ethyl methanesulphonate	0·33	0·52	A, B	Roberts & Warwick (1958)
Sulphobromophthalein	0·19	0·35	A, B	—
1:2-Epoxy-1:2:3:4-tetrahydronaphthalene	0·49	0·63	A, B	Boyland & Sims (1960)
Iodobenzene*	0·40	0·56	B	Hele (1924); Mills & Wood (1953)
Naphthalene*	0·37†	0·47†	A, B	Bourne & Young (1934)
1-Chloronaphthalene*	0·49	0·65	B	Cornish & Block (1958)
Phenanthrene*	0·43† 0·38‡	0·61† 0·57‡	B	—

* Required microsomes and $TPNH_2$.
† Gave blue colours with diazotized *p*-nitroaniline and Na_2CO_3 (reagent 3).
‡ Gave orange colours with reagent (4).

oxynaphthalene on paper chromatograms. Phenanthrene yielded two compounds indistinguishable from *trans*-1:2-dihydro-1:2-dihydroxy- and *trans*-9:10-dihydro-9:10-dihydroxy-phenanthrene, which gave blue and orange colours respectively on paper chromatograms treated with reagent 4.

DISCUSSION

The detoxication of foreign compounds, such as the monohalogenobenzenes (Baumann & Preusse, 1879; Jaffé, 1879), as mercapturic acids has been known for many years, and several GSH compounds have been suggested as the source of the cysteine residues. The possibility that GSH was involved in the detoxication of avertin was suggested by Waelsch (1930). Administration of naphthalene to rabbits caused a decrease in the GSH content of the eye lens and liver (Nakashima, 1934) and Yamamoto (1940) found that when bromobenzene was given to rabbits there was a decrease in the GSH level in several organs, particularly liver, which was paralleled by the amount of *p*-bromophenylmercapturic acid excreted. Bromobenzene, when given to rats, caused an increase in GSH levels in the liver and kidney 30 min. after administration, followed by a decrease in the levels lasting for 2 days, and it was suggested that this decrease was due to the formation of sulphuric acid esters and a mercapturic acid (Binet & Wellers, 1951). Barnes *et al.* (1959) gave a variety of mercapturic acid precursors to rats and found falls in the liver GSH level after the administration of the compounds; these falls could be related to the amounts of mercapturic acids formed. One of these compounds was 3:4-dichloronitrobenzene, and it had been shown that rabbits dosed with this substance excreted the mercapturic acid, *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine (Bray *et al.* 1957). The present work has shown that the biosynthesis of this metabolite can be carried out by tissue preparations in the three stages suggested by Bray *et al.* (1959*b*). The first reaction, which is catalysed by the soluble fraction of rat liver, is the replacement of the *para* chlorine atom by GSH. Since the activity of the fraction is destroyed by heat and is not affected by dialysis, it appears to be due to an enzyme which has no absolute cofactor requirements. The second stage is the conversion of *S*-(2-chloro-4-nitrophenyl)glutathione into *S*-(2-chloro-4-nitrophenyl)-L-cysteine by kidney homogenate, a reaction which is probably due to two enzymes previously found in pig kidney and which catalyse the removal of the glutamyl group from glutathione itself and the hydrolysis of cysteinylglycine (Olson & Binkley, 1950). That glutathione derivatives can also behave in this way has been shown by the conversion of *S*-(*p*-chlorobenzyl)-glutathione into *S*-(*p*-chlorobenzyl)-L-cysteine by liver slices of various species (Bray *et al.* 1959*a*). In the third stage, rat-liver slices were used to demonstrate the *N*-acetylation of *S*-(2-chloro-4-nitrophenyl)-L-cysteine to the mercapturic acid, and again other similar reactions have been reported, such as the *N*-acetylation of *S*-benzyl-DL-homocysteine by rat-liver and -kidney slices (Gutmann & Wood, 1951) and of *S*-(*p*-chlorobenzyl)-L-cysteine by liver slices (Bray *et al.* 1959*b*). The conversion of GSH derivatives into mercapturic acids *in vivo* has been demonstrated by Roberts & Warwick (1958), who found that when *S*-ethylglutathione was administered to rats it was excreted as *S*-ethylmercapturic acid, and Stekol (1940, 1941) showed that administration of *S*-benzylglutathione and *S*-(*p*-bromobenzyl)glutathione to rats resulted in the excretion of *N*-acetyl-*S*-benzyl-L-cysteine and *N*-acetyl-*S*-(*p*-bromobenzyl)-L-cysteine respectively.

The enzyme catalysing the reaction in which the *para* chlorine atom of 3:4-dichloronitrobenzene is replaced by GSH appears to be specific for GSH, but not for the other substrate, since it was found that GSH could not be replaced by GSSG, cysteine or *N*-acetylcysteine, whereas other compounds could replace 3:4-dichloronitrobenzene. Many of these are known to be converted into mercapturic acids in the body (see Table 5). With 2:4- and 3:4-dichloronitrobenzene (which yield mercapturic acids in which chlorine atoms are replaced by *N*-acetylcysteine), and 2:3:5:6-tetrachloronitrobenzene (which yields a mercapturic acid by the replacement of the nitro group by acetylcysteine), no GSH derivative was obtained if the soluble liver fraction was boiled before the incubation, indicating that these reactions were enzymic. 3:4:5:6-Tetrachloro-1:2-epoxy*cyclo*hexane also reacted with GSH only in the presence of the soluble liver fraction.

With the substrates bromoethane, 1-bromopropane, benzyl chloride, ethyl methanesulphonate and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, all of which are alkylating agents, reactions with GSH both in the presence of fresh and of boiled soluble liver fraction was observed although the first reaction was much faster. These reactions were carried out at pH 8, which is the optimum pH of the enzyme for 3:4-dichloronitrobenzene, so that the observed chemical reaction, presumably requiring ionized SH groups of GSH, is not unexpected. Sulphobromophthalein, which also reacted with GSH both in the presence and the absence of the enzyme, is used as a test substance for liver function because it is removed from the blood by the liver and it is excreted in the bile partly in the form of a GSH conjugate (Javitt, Wheeler, Baker & Ramos, 1959; Combes & Stakelum, 1960). It is possible that this liver-

function test is actually a measure of the activity of the enzyme.

The substrates iodobenzene, naphthalene, 1-chloronaphthalene and phenanthrene did not react with GSH in the presence of soluble liver fraction alone, but only if microsomes and $TPNH_2$ were also present. With naphthalene, one of the products thus obtained was indistinguishable on paper chromatograms from *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione, which has been previously identified as a metabolite of naphthalene formed by liver slices (Booth *et al.* 1960*b*) and in the bile of rats dosed with naphthalene (Boyland, Ramsey & Sims, 1961). The second product was *trans*-1:2-dihydro-1:2-dihydroxynaphthalene, which has been reported as a product of the action of microsomes and $TPNH_2$ on naphthalene (Mitoma, Posner, Reitz & Udenfriend, 1956; Booth & Boyland, 1957). Phenanthrene gave two GSH conjugates, and also yielded *trans*-1:2-dihydro-1:2-dihydroxy- and *trans*-9:10-dihydro-9:10-dihydroxy-phenanthrene, which are known metabolites in urine (Boyland & Wolf, 1950).

When 1:2-dihydronaphthalene is incubated with rat-liver microsomes and $TPNH_2$, perhydroxylation occurs with the formation of *trans*-1:2:3:4-tetrahydro-1:2-dihydroxynaphthalene, whereas if GSH is added *S*-(1:2:3:4-tetrahydro-1-hydroxy-1-naphthyl)glutathione is also formed. If rat soluble liver fraction is also present the amounts of the glutathione conjugate formed are much increased (Booth *et al.* 1960*a*). It was suggested that in these reactions the action of microsomes and $TPNH_2$ was to convert 1:2-dihydronaphthalene into 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, which then reacted with GSH, either with or without the enzyme present in the soluble liver fraction, to yield the GSH conjugate. It seems probable that, in the present work, the four compounds requiring microsomes and $TPNH_2$ in addition to the enzyme are first converted into similar intermediates. With these compounds, however, the presence of the soluble liver fraction is usually necessary for the formation of the GSH conjugates, although occasionally small amounts of the GSH conjugate derived from naphthalene, presumably formed by a chemical reaction with the intermediate, have been detected in experiments in which boiled soluble liver fraction has been used.

Fig. 4 shows in effect that compounds which are apparently alternative substrates for the enzyme inhibit the reaction of GSH with 3:4-dichloronitrobenzene non-competitively. This indicates that these compounds are combining with the enzyme at a point other than the active centre for 3:4-dichloronitrobenzene and would seem to imply that the enzymic reactions involved are different so far as active centres are concerned. It seems likely that the enzyme involved in these reactions can produce an activated form of GSH which then reacts more readily with certain compounds. However, since the dialysed soluble liver fraction was used to catalyse the enzymic reactions shown in Table 5, the possibility that all these GSH conjugates are not formed by the same enzyme is not completely eliminated.

SUMMARY

1. An enzyme which catalyses the formation of *S*-(2-chloro-4-nitrophenyl)glutathione from 3:4-dichloronitrobenzene has been partially purified from the soluble fraction of rat liver.

2. *S*-(2-Chloro-4-nitrophenyl)glutathione is converted into *S*-(2-chloro-4-nitrophenyl)-L-cysteine by rat-kidney homogenate, and this compound can be acetylated to the mercapturic acid, *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine by rat-liver slices.

3. The soluble liver fraction can catalyse the formation of glutathione conjugates from a number of other mercapturic acid precursors.

4. With bromobenzene, naphthalene, 1-chloronaphthalene and phenanthrene, rat-liver microsomes and reduced triphosphopyridine nucleotide as well as the soluble liver fraction are necessary in the formation of the glutathione conjugates.

5. Glutathione cannot be replaced by oxidized glutathione, cysteine or *N*-acetylcysteine in these systems.

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The Intrinsic Viscosity of Myosin and the Interpretation of its Hydrodynamic Properties

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Early work suggested a particle weight of about 800 000 for the myosin molecule, with an axial ratio (J) of 100:1 (Portzehl, 1950), but more recently it has appeared that some of the data employed were in error. For the sedimentation coefficient, a detailed account has been given of the probable cause of this error in terms of molecular-transformation reactions occurring during the experiments (Johnson & Rowe, 1960). There may well have been similar errors in much recent as well as early diffusion work; also in viscosity, light-scattering and osmotic-pressure experiments. There has as yet, however, been no general agreement upon revised estimates of the size and shape of the myosin molecule.

For the complete definition of an ellipsoid of revolution hydrodynamically equivalent to the myosin molecule, three independent (usually kinetic) parameters must be known, together with the partial specific volume ($\bar{v}$). The method of Scheraga & Mandelkern (1953) then provides a convenient means for the calculation of the axial ratio and the effective volume/g. (V) of this ellipsoid. Let us take the extrapolated sedimentation and diffusion coefficients, $[S^0_{20}]$ and $[D^0_{20}]$, and the intrinsic viscosity, $[\eta]$, as the three kinetic parameters. $[S^0_{20}]$ has been accurately determined under conditions where myosin is known to be stable (Holtzer & Lowey, 1956, 1959; Johnson & Rowe, 1960), as also has $[D^0_{20}]$ (Parrish & Mommaerts, 1954), although the value given by Parrish & Mommaerts should be confirmed by an independent and equally thorough study. But the values given in the literature for $[\eta]$ show a surprising degree of scatter, the following being among those reported (dl./g.): 2·2 (Mommaerts, 1945, and Weber, 1950*a*, *b*); 1·8–2·0 (Weber & Portzehl, 1951; Portzehl, Schramm & Weber, 1951); 2·3, 2·2 and 2·4 (Holtzer & Lowey, 1956). Since the completion of this work Holtzer & Lowey (1959) have reported a value of 2·17 at 25°, and a rather higher value (2·24) at 0·7°. In view of the occur-

rence of a fair degree of scatter, Holtzer & Lowey do not consider this difference to be significant.

In the work here reported the difficulties in the accurate viscometry of myosin solution have been to a large extent overcome by the use of a modified viscometer and a carefully defined technique. An accurate value for $[\eta]$ is reported, which is used in calculating the dimensions of the equivalent ellipsoid of revolution.

EXPERIMENTAL

Materials

Water. All water used was thrice-distilled, as previously described (Johnson & Rowe, 1960).

Reagents. All reagents used were of A.R. quality.

Buffer solution. All experiments were performed in a stock buffer solution, pH 6·7 and *I* 0·60, having the following composition (g./l.): KCl, 41·0; $Na_2HPO_4,12H_2O$, 4·0; KH_2PO_4, 2·2.

Myosin. Myosin was prepared from rabbit skeletal muscle as previously described (Johnson & Rowe, 1960). The purified protein was stored in stock buffer solution at 2–4° in sealed, sterile flasks.

Methods

Viscosity determinations. All viscosity determinations were performed with capillary viscometers reproducibly located on rigid, detachable mounts in a lagged water thermostat equipped with cooling coils. The temperature could be maintained to within ±0·02° over the range 5–25°.

Simple Ostwald and Ubbelohde viscometers, maintained at 20°, were used originally, but were found to give very poorly reproducible results with myosin solutions. In the light of experience gained of the general properties of myosin, the following peculiar difficulties can be listed:

(i) Myosin undergoes transformation reactions at room temperatures, and these are accompanied by viscosity changes (Lowey & Holtzer, 1959*a*; P. Johnson & A. J. Rowe, in preparation).

(ii) 'Threads' of denatured protein readily form in the bulk of the solution, and obstruct flow through the capillary.

(iii) A slight amount of surface foam is very easily formed during manipulation of solutions. Accurate measurements require the observation of a clean, well-defined meniscus.

(iv) Myosin has a tendency to precipitate on glass surfaces (Rupp & Mommaerts, 1957). Even if the bulk concentration is little affected, a film of protein may line the capillary, both narrowing the bore and also tending to entrap air bubbles during re-charging of the viscometer.

The problem of capillary adsorption is discussed below. The remaining difficulties have been overcome by the use of a modified viscometer and by working at 5±0·02° with a rigidly defined technique.

The modified capillary viscometer used was similar to one described by Nawab, Goring & Mason (1957) and is illustrated in Fig. 1. Described as a 'non-foaming' viscometer, it is so designed that where a certain amount of surface foam appears during manipulation this can be spilled over into the upper reservoir A by suction at C, with the connecting tube F sealed off by the three-way tap E. The detailed technique of measuring and dilution was as described by Nawab *et al.* (1957). Briefly, the viscometer was charged from the lower reservoir B by suction at C, allowing solution to overspill into A until the lower meniscus reached the mark Z: the fall of the upper meniscus between the levels X and Y was then timed. The capillary was at all times kept filled with solution, and the lower bulb Q was used solely to prevent the meniscus from entering the capillary after each reading. Dilutions were made in the reservoir B, tap E being opened to drain the upper reservoir. The efficiency of the dilution procedure was checked by control experiments with coloured fluids. For experiments with variable shear-rate, a system of variable large-capacity reservoirs was connected at C, the resulting constant pressures being read from a water manometer by means of a cathetometer.

After normal cleaning (chromic acid; water; A.R. acetone) the viscometer was dried by sucking through it a stream of filtered air. Solutions were freed from dust and protein 'threads' by filtration under gravity through cotton-wool plugs. This method was found to be superior to the use of removable sintered-glass tips as filters on pipettes used in transfer, a technique which was also tried but abandoned as liable to produce foaming.

Values for flow-times were accepted only if the capillary was visibly free from obstruction and if several readings consistent to ±0·15 sec. (< 0·2 g./100 ml.) or to ±0·25 sec.

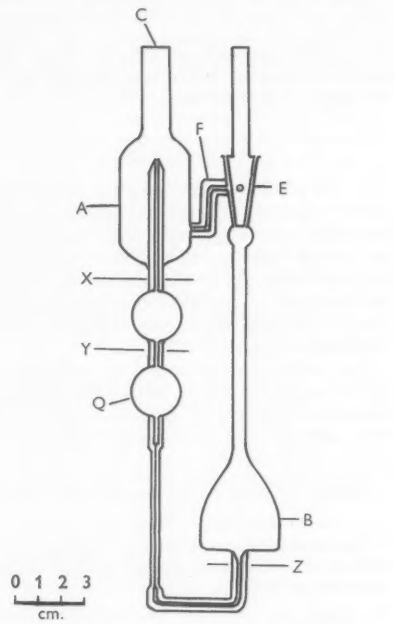

Fig. 1. 'Non-foaming' capillary viscometer used for myosin solutions. Capillary length, 6·3 cm.; internal diam. 0·05 cm. (approx.). Minimal charging volume, 10 ml. For description of parts see the Experimental section.

(higher concentrations) could be obtained. By these criteria results from second and subsequent dilutions were invariably rejected, and often those from first dilutions also. Latterly, dilution *in situ* has been abandoned, and the viscometer cleaned, dried, filled and equilibrated for each concentration. Acceptable results were then usually obtained.

The kinetic characteristics of the viscometer were determined by the method described by Merrington (1949). For a given viscometer

$$\eta = \rho[At - (B/t)], \quad (1)$$

where η is the dynamic viscosity of the fluid under investigation, ρ is the density of the fluid, t is the flow time and A and B are constants. The term B/t represents a correction for the kinetic energy of the fluid leaving the capillary.

Water and aqueous glycerol solutions, whose exact composition was found from density determinations, were employed as standards. Details of the density and viscosity of aqueous glycerol solutions were taken from the tables of Bosart & Snoddy (1927) and Sheely (1932). The results obtained are shown in Table 1. The quantity B is seen to be too small to be measured with any accuracy. For a typical experiment with a myosin solution, with t 250 sec., the correction term (B/t) amounts to 0·2% of (At), taking B 0·9. Because of the smallness of this correction and the uncertainty of its exact value, it has not been applied to the data reported.

Measurements of protein concentration. Portions of solution were removed from the viscometer after each experiment, and the nitrogen content was estimated by the micro-Kjeldahl method (Pregl & Roth, 1945). A nitrogen content of 16·2% was assumed for myosin (Mommaerts & Parrish, 1951).

RESULTS

It was shown by Mommaerts (1945) that the relative viscosity of dilute myosin solutions shows little shear-rate dependence. This was confirmed in the present work by following flow-times at various pressure-heads and several concentrations (Fig. 2). The linearity of the plots demonstrates that the effect of varying shear was negligible under the conditions employed.

The collected data from four preparations are shown in Figs. 3 and 4. Above a protein concentration of 0·2 g./100 ml. both the relative viscosity ($\eta_{rel.}$) and the reduced specific viscosity ($\eta_{sp.}/c$)

Table 1. *Values for the characteristic constants A and B*

Values are for the 'non-foaming' capillary viscometer, determined by the use of aqueous glycerol solutions and water as standards.

Concn. of glycerol soln. used (%)	*A*	*B*
5·45	0·00719 (±0·00011)	0·4 (±0·6)
12·05	0·00724 (±0·00007)	1·4 (±0·6)

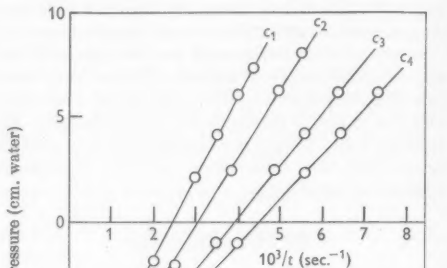

Fig. 2. Reciprocal flow-times of myosin solutions at 20° with various pressure heads. Concentrations: c_1, 0·46; c_2, 0·42; c_3, 0·28; c_4, 0·21 g./100 ml. Solutions in stock buffer solution.

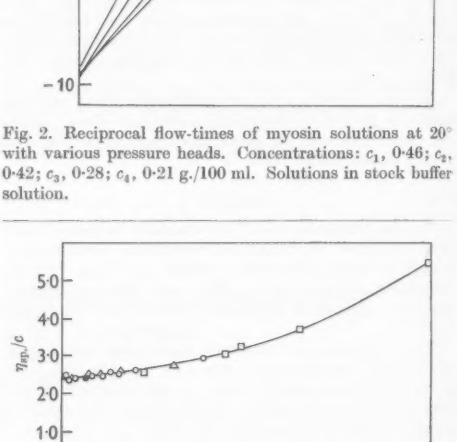

Fig. 3. Reduced specific viscosity of myosin in stock buffer solution as a function of concentration. □, Myosin VI; ○, myosin VII; △, myosin VIII at 5°; ●, myosin IX at 25°, extrapolated to zero time of incubation.

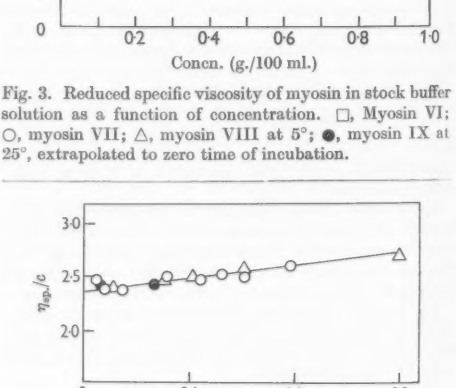

Fig. 4. Reduced specific-viscosity data for myosin at concentrations below 0·3 g./100 ml. Symbols are defined in Fig. 3.

cease to be linear with respect to concentration, as noticed by Szent-Gyorgyi (1951). Below this concentration, the relation between $\eta_{sp.}/c$ and c is linear to a good approximation. The line drawn was determined by the method of least squares for the data from myosin VII and myosin VIII (Fig. 4). From the intercept, a value for the intrinsic viscosity $[\eta] = 2{\cdot}35 \pm 0{\cdot}01$ dl./g. has been obtained. Further uncertainty arises from doubts about the nitrogen content of the protein. Thus Dr K. Bailey (personal communication) considers the value 16·7% to be more correct than that (16·2%) assumed here, and use of the higher figure would yield an intrinsic viscosity of 2·42. The result given here falls within the range of values obtained by previous workers (also subject to uncertainty in the nitrogen content), and the problem of reproducibility appears to have been successfully overcome.

Two results obtained with myosin IX at 25° are included in Figs. 3 and 4. As is shown by P. Johnson & A. J. Rowe (in preparation), the viscosity of myosin solutions increases with time at this temperature, and these results have been obtained by extrapolation of values of $\eta_{sp.}/c$ to zero time of incubation. The concordance with results at 5° provides further evidence that the hydrodynamic behaviour of native myosin is consistent over this temperature range (cf. Johnson & Rowe, 1960).

DISCUSSION

Is capillary adsorption occurring? The possibility of adsorption of protein on the walls of the capillary has not been specifically ruled out in the above-described experiments. Moreover, the fact that values for $\eta_{sp.}/c$ as determined at low concentration lie above the line which might be extrapolated from higher concentration ($> 0{\cdot}4$ g./100 ml.) might suggest such an adsorption. However, two sets of considerations make it very unlikely that $[\eta]$ is in error for this reason.

First, Öhrn and others have described the effect of capillary adsorption theoretically, and observed it empirically in solutions of synthetic polymers in organic solvents (Öhrn, 1955, 1956; Tuijnman & Hermanns, 1957; Takeda & Endo, 1956; Rutherford & Pepper, 1959; Huque, Fishman & Goring, 1959). Their interpretation has, however, been disputed by Boyer & Streeter (1955). Experimentally, a steep rise in the reduced specific viscosity as the concentration falls has been generally found, followed by a 'fall-over' and steep drop as the concentration approaches zero. This differs markedly from the behaviour of myosin solutions.

Secondly, for adsorption to occur it would have to be: (i) Complete within equilibration time. At 5° the viscosity of myosin solutions does not change with standing in the viscometer. (ii) Completely reproducible between different chargings of the cleaned viscometer, different preparations and different temperatures (5° and 25°). (iii) Preferential on the capillary walls, rather than on the bulbs and reservoirs of the viscometer.

Postulate (i) is quite reasonable: (ii) and (iii) are most unlikely to be true. Even allowing for immobilized solvent contributing to the adsorbed layer, it may be calculated that the total amount of protein present at the lowest concentration (about 1 mg.) is quite insufficient to provide adequate generalized adsorption. Further, at low protein concentrations ($< 0{\cdot}2$ g./100 ml.), adsorption if appreciable should be directly observable as a significant fall in the solution concentration. Only small decreases have been observed experimentally. Moreover, after storing myosin solution in a cleaned viscometer, and then carefully draining, we have been unable to demonstrate anything more than a very small ($< 0{\cdot}5$%) rise in flow-times for either protein solution or solvent over that obtained with a clean viscometer, unless gross visible obstruction of the capillary had occurred. Thus positive evidence for capillary adsorption is lacking, and indirect evidence strongly suggests that it was not an important factor in these experiments.

However, it should be borne in mind that generalized adsorption of protein on the viscometer walls could cause a drop in bulk protein concentration even though flow-times were but little affected. Thus during filling and charging of the viscometer, a solution (10 ml.) is exposed to at least 100 cm.^{2} of glass surface. A uniformly adsorbed film 10^3 Å thick would deplete the protein content by 1·3 mg., although the flow time for a capillary of diameter 0·05 cm. would be increased by only 0·16%. Thus the protein concentration should be measured after equilibration in the viscometer, the only remaining source of possible error then being adsorption on the pipette during the transfer to the Kjeldahl-digestion flask.

Comparison with other values for $[\eta]$. Weber's low value for $[\eta]$ (Portzehl *et al.* 1951) may be ascribed to the fact that it was obtained from results at concentrations greater than 0·2 g./100 ml. The present value falls within the range of other values quoted to within the various experimental errors: however, it appears to be just significantly higher than the most recent value given by Holtzer & Lowey (1959) ($2{\cdot}17 \pm 0{\cdot}1$ dl./g. at 25°). Probably differences in techniques of concentration measurement account for this discrepancy. Holtzer & Lowey obtained c values by dilution from stock solution: as is described above, adsorption on the walls of the dilution vessels and of the viscometer could result in these c values

being erroneously high, and hence in low values for $\eta_{sp.}/c$. These latter values might well decrease with increasing temperature, as described by Holtzer & Lowey, since surface denaturation and adsorption would be expected to increase with increasing temperature. For these reasons, and also because the Ostwald–Fenske viscometers used by Holtzer & Lowey are less well suited to the system and give a greater scatter of results than the 'non-foaming' viscometer, we consider that the present value for $[\eta]$ is to be preferred.

Calculation of the size and shape of the equivalent ellipsoid. It was shown by Scheraga & Mandelkern (1953) that the axial ratio of the equivalent ellipsoid may be calculated from a single function, β, defined by

$$\beta = N.\eta \left(\frac{[S]\,[\eta]}{(1-\bar{v}\rho)}\right)^{\frac{1}{3}} \left(\frac{[D]}{RT}\right)^{\frac{2}{3}}, \qquad (2)$$

where η is the viscosity of the solvent, ρ is the density of the solution and $[S]$, $[\eta]$, $[D]$ and $\bar{v}$ are respectively the extrapolated sedimentation coefficient, reduced specific viscosity, diffusion coefficient and partial specific volume of the solute, under a given set of conditions. For myosin, the above-mentioned quantities (with the possible exception of $[D]$) have recently been measured with accuracy at low temperatures where myosin is stable. The solvents used were closely similar in composition, and it has been shown that small variations in ionic strength, composition and pH do not affect the sedimentation or light-scattering behaviour of myosin solutions (Parrish & Mommaerts, 1954; Holtzer & Lowey, 1959; Johnson & Rowe, 1960, and present work). The intrinsic viscosity of the native protein does not appear to vary with temperature over the range 5–25°, and it has already been shown that this is also true for the sedimentation coefficient over the range 3–19° (Holtzer & Lowey, 1959; Johnson & Rowe, 1960). Thus we can be reasonably confident that all the data refer to identical or at least equivalent experimental conditions. Substituting $[S^0_{20}] = 6{\cdot}43$ s, $[\eta] = 2{\cdot}35$ dl./g., $\bar{v}_{20} = 0{\cdot}725$, $[D^0_{20}] = 1{\cdot}05 \times 10^{-7}$ c.g.s.u. and values for η^0_{20} and ρ^0_{20}, a value $\beta = 2{\cdot}83 \pm 0{\cdot}06$ is yielded. An axial ratio $J = 34{\cdot}5 \pm 5$ is obtained by interpolation from Scheraga & Mandelkern's tables of the function: this corresponds to an effective volume $V = 2{\cdot}52$ ml./g. (by application of the equation of Simha, 1940). A possible error of $\pm 2\%$ has been assumed for the diffusion coefficient: other errors in the evaluation of β are relatively small.

As all the data were obtained at a high ionic strength, the possible effect of selective solvation on the term $(1-\bar{v}\rho)$ must be considered (Schachman & Lauffer, 1950; Brand & Johnson, 1958). Assuming (as an outside estimate) that 0·5 ml. of water is selectively associated with 1·0 g. of dry protein, then the term $(1-\bar{v}\rho)$ must be replaced by $1-\bar{v}\rho+0{\cdot}5\ (\rho_{water}-\rho)$, and β becomes 2·88. This increase in β is within the cumulative experimental uncertainty, and the effect of selective solvation on β is not further considered.

The effective volume of the equivalent ellipsoid is large, suggesting that a considerable amount of solvent may be associated with the molecule. Correspondingly, the axial ratio is lower than that which would be calculated assuming zero solvation for the molecule from $[\eta]$ and $\bar{v}$ ($J = 70$), or from $[D^0_{20}]$, $[S^0_{20}]$ and $\bar{v}$, which yield a value $J = 86$. These latter values, which involve the assumption of $V = \bar{v}$, are in good agreement with those derived by Holtzer & Lowey (1959), using the same assumption, from the equations of Riseman & Kirkwood (1950) and Kirkwood & Auer (1951), which treat the molecule as a rigid string of spherical beads.

An independent estimate of the size and shape of the hydrodynamic particle is clearly desirable. The theory derived by Fessler & Ogston (1951) and Ogston (1953) for the dependence of S^0_{20} upon concentration offers the possibility of obtaining such an estimate from the parameters $\mathrm{d}\left(\frac{1}{S}\right)\Big/\mathrm{d}c$, $[S^0_{20}]$, $[\eta]$ and $\bar{v}$. It has been found that the variation of S^0_{20} with concentration for myosin can be well described in terms of this theory; allowance being made for the fact that as the concentration approaches zero, one of the constant terms (k) in Fessler & Ogston's equations itself varies with concentration, an effect to be expected from earlier work by Emersleben (1925) and Sullivan (1941) but not incorporated in Fessler & Ogston's treatment. A value for $\mathrm{d}(1/S)/\mathrm{d}c = 0{\cdot}0956$ has been measured, mean sedimenting concentrations being used as suggested by Kegeles & Gutter (1951). Assuming values $k = 3$, $\zeta = 0{\cdot}67$ for the constant terms, values of $M = 543\,000$, $J = 32{\cdot}7$, $V = 2{\cdot}71$ have been obtained as described by Ogston (1953). The selection of values for k and ζ is to a considerable extent arbitrary. For sedimenting protein systems Ogston (1953) used the value $k = 1{\cdot}7$ with initial protein concentrations in calculating values of $\mathrm{d}(1/S)/\mathrm{d}c$. If his results are recalculated by using estimated mean concentrations during sedimentation they are no longer decisive in favouring a value lower than $k = 3$. The latter value is supported also by calculation for model systems (Emersleben, 1925). With initial concentrations in sedimentation and $k = 1{\cdot}7$, $\zeta = 0{\cdot}67$ (Ogston, 1953), then we obtain $J = 26{\cdot}4$, $M = 565\,000$. The agreement with the previous results is very satisfactory, but it should be emphasized that the Fessler–Ogston treatment is to a considerable extent an empirical one, and too much significance should not therefore be attached to the extent of this agreement.

Molecular weight of myosin. With the data given above, the sedimentation–diffusion method yields a value $M = 540\,000 \pm 15\,000$. This agrees reasonably well with the most careful estimates by the light-scattering method (Rupp & Mommaerts, 1957; Holtzer & Lowey, 1956, 1959). However, recent estimates by the 'Archibald' technique have yielded values as low as 420 000 (Mommaerts & Aldrich, 1958; Hippel, Schachman, Appel & Morales, 1958; Lowey & Holtzer, 1959*b*), and this lower value has been quoted as the best available estimate for M in several reviews of the field (e.g. Morales, 1960; Huxley, 1960).

This discrepancy is not easily explained. Selective solvation to the extent described earlier reduces the sedimentation–diffusion value for M to 514 000, but then the 'Archibald' values should be corrected in exactly the same ratio. It does not seem likely that the values for $[S^0_{20}]$, $[D^0_{20}]$ and $\bar{v}$ now used could be in cumulative error to the extent of 29 %, as is required by acceptance of the lowest 'Archibald' values. In fact these various quantities now seem to be known to a higher degree of precision, over a range of conditions, than for the great majority of 'well-defined' proteins. The chief remaining source of uncertainty lies in the value for $[D^0_{20}]$ obtained by Parrish & Mommaerts, which has not been independently confirmed; but values obtained by other workers have all been lower rather than higher (Weber, 1950*a*; Laki & Carroll, 1955).

Using an alternative expression for the β coefficient (Scheraga & Mandelkern, 1953)

$$\beta = \frac{N[S]\eta[\eta]^{\frac{1}{3}}}{M^{\frac{2}{3}}(1-\bar{v}\rho)}, \qquad (3)$$

and the lowest 'Archibald' value for M, an equivalent ellipsoid may be constructed independently of a value for the diffusion coefficient. The results yielded are: $\beta = 3{\cdot}34$, $J = 143$, $V = 0{\cdot}2$.

A recent comprehensive study in this Laboratory of published hydrodynamic data for proteins has shown that there is probably no well-established case where the effective volume is substantially smaller than the partial specific volume (E. G. Richards, personal communication). The J value obtained is also greatly in excess of the maximum permissible value calculated directly from the Simha equation assuming zero hydration. Thus the weight of evidence suggests that the low 'Archibald' values for M are in error, but the reason for this is not clear. Errors in determination of the meniscus position as described by Trautman (1958) as well as the inherent large uncertainty of the method are probably involved.

Conclusions. If the anomalous results yielded by the 'Archibald' method can be explained, then the behaviour of the myosin molecule in solution now seems to be as well defined as current hydrodynamic theory will permit. In addition to a comprehensive diffusion study, the chief additional data which might usefully be obtained are on the flow–birefringence at low temperatures, the experiments of Joly, Schapira & Dreyfus (1955) being difficult to interpret since these were obtained at room temperatures.

If, as is usually thought, the myosin molecule is indeed a rigid, elongated structure, then a prolate ellipsoid of revolution should provide a fair model, and we may tentatively interpret our equivalent ellipsoid in physical terms. Its most striking feature is its large effective volume. This cannot be explained by normal estimates of solvation, and might be accounted for either in terms of a 'swept' or 'excluded' volume caused by rotation of an asymmetrical particle, or else in terms of a volume of solvent included 'within' the molecule. Since extensive solvent immobilization within the tertiary structure is unlikely, this latter possibility would correspond to a structure such as a hollow cylinder, the solvent within the cylinder being postulated as contributing to the hydrodynamic volume. Taking 1620 Å as the molecular length (Holtzer & Lowey, 1959), the axial ratio as 34·5 (for high axial ratios, prolate ellipsoids and cylinders of revolution are indistinguishable) and $M = 540\,000$, $\bar{v} = 0{\cdot}725$, a value $V = 3{\cdot}2$ is yielded for such a hollow cylinder, in reasonable agreement with that found for the equivalent ellipsoid. Electron-microscope studies are likely to offer the most direct further evidence on this problem, and an investigation by this technique is under way. Valentine (1960) has shown that the seed protein, legumin, appears as a hollow cylinder in electron micrographs.

SUMMARY

1. The problems involved in the viscometry of myosin solutions have been investigated, and many of the difficulties overcome by the use of a modified form of capillary viscometer. A value for the intrinsic viscosity of myosin,

$$[\eta] = 2{\cdot}35 \pm 0{\cdot}03 \text{ dl./g.},$$

is reported. With this value, together with

$$[S^0_{20}] = 6{\cdot}43\text{s}, \; [D^0_{20}] = 1{\cdot}05 \times 10^{-7} \text{ c.g.s.u.},$$
$$\bar{v} = 0{\cdot}725,$$

a β factor of 2·83 is calculated. The hydrodynamically equivalent ellipsoid possesses an axial ratio $J = 34{\cdot}5$ and effective volume $V = 2{\cdot}52$ ml./g. An independent treatment utilizing the sedimentation–concentration dependence is shown to give consistent results.

2. The values for the molecular weight obtained by the 'Archibald' method are shown to be inconsistent with the present data, and it is suggested that the Archibald values may be too low.

3. The properties of the equivalent ellipsoid are discussed in terms of possible molecular models, and the dimensions of one model, a hollow cylinder of revolution, are calculated.

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Studies on the Metabolism of the Chrysophyceae

COMPARATIVE STRUCTURAL INVESTIGATIONS ON LEUCOSIN (CHRYSOLAMINARIN) SEPARATED FROM DIATOMS AND LAMINARIN FROM THE BROWN ALGAE

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The Chrysophyceae comprise a large number of plankton and benthic algae of fresh and sea water; many, if not all, of the diatoms belonging to this Order synthesize an intracellular polysaccharide, described by the phycologists as leucosin. This carbohydrate occurs dissolved in the cell vacuole, the refractive index of the latter being proportional to its leucosin content. Pringsheim (1952) is, however, of the opinion that the term leucosin has been applied to very different substances; indeed this polysaccharide has been variously described as a polyfructose resembling inulin and a polyglucose similar to laminarin. Stosch (1951) separated an insoluble form of leucosin from freshwater diatoms and both an insoluble and soluble form of this polysaccharide from marine diatoms (H. A. von Stosch, unpublished communication to the Third International Seaweed Symposium, Galway, 1958). This author considers that, in view of its solubilities and the chromatographic behaviour of the derived mono- and di-saccharides, leucosin is closely related to the soluble and insoluble forms of laminarin, essentially linear β-1:3′-linked glucans synthesized by various species of the brown marine algae (Phaeophyceae). A polysaccharide termed leucosin has also been separated from the flagellated protozoon *Ochromonas malhamensis*. Archibald, Manners & Ryley (1958) have established that this material resembles laminarin in that it, too, consists of β-1:3′-linked glucose units, with a small proportion of other linkages also present.

The difficulty of isolating sufficient leucosin from the Chrysophyceae has precluded systematic structural investigations on this polysaccharide. Quillet (1955) found that leucosin extracted from *Hydrurus foetidus* had $[\alpha]_D - 6°$, gave no stain with iodine and chromatographic examination of an acid hydrolysate revealed that glucose was the only sugar present. A paper chromatogram of a partial acid hydrolysate showed six evenly spaced spots together with several spots near the starting line, from which Quillet concluded that the polysaccharide contained at least eight anhydroglucose units. In view of the ambiguity of the term leucosin this author suggests that a better name for this material would be chrysolaminarin. The recent development of new techniques has made possible structural investigations of polysaccharides on milligram quantities and this paper describes the results obtained from 0·5 g. of crystalline (insoluble) leucosin, hereinafter called chrysolaminarin. This material was separated from a mixture of diatoms belonging to the Chrysophyceae, and was placed at the disposal of the authors through the generosity of Professor H. A. von Stosch. In view of the limited supply of polysaccharide, and of its possible identity with the laminarin isolated from the brown seaweeds, it appeared advisable to carry out parallel experiments on insoluble laminarin isolated from *Laminaria cloustoni*. The similarities and differences between the two polysaccharides are discussed.

MATERIALS AND METHODS

Analytical methods

Chromatography. (*a*) Qualitative. Descending paper chromatograms were prepared at room temperature with Whatman no. 1 paper and butanol–pyridine–benzene–water (5:3:1:3, by vol.) (A), butan-2-one (ethyl methyl ketone)–acetic acid–water saturated with boric acid (9:1:1, by vol.) (B), ethyl acetate–pyridine–water (10:4:3) (C), ethyl acetate–acetic acid–formic acid–water (18:3:1:4) (D), butanol–ethanol–water (4:1:5) (E), butan-2-one–water (10:1) (F) and butanol–pyridine–water (10:3:3) (G) as solvents. Aniline oxalate (i), silver nitrate–sodium hydroxide (ii) [for details of (i) and (ii) see Bell (1955)], periodate–benzidine (iii) (Cifonelli & Smith, 1954), bromocresol green (iv) (0·1% soln. in 95% ethanol made alkaline with sodium hydroxide) were used as spray reagents. The rate of movement of sugars is expressed relative to tetra-*O*-methylglucose (R_G values) or to glucose (R_{Gl} values).

(*b*) Quantitative. The methylated sugars were estimated by the method of Schaefer & Van Cleve (1956).

Electrophoresis. Separation on Whatman no. 1 paper was effected in an apparatus described by Foster (1952) at 750 V and 10 mA. 0·1 M-Borate buffer, pH 10·0, was used.

Acid hydrolysis. (*a*) Complete acid hydrolysis was carried out by heating the polysaccharide with N-sulphuric acid (1·5% soln., w/v) at 100° until the rotation was constant (6 hr.). Reducing sugar was determined with the Somogyi (1952) reagent calibrated against glucose.

(*b*) Partial acid hydrolysis was effected by heating with 0·1 N-sulphuric acid (0·5% soln., w/v) for 1 hr. at 100°.

(*c*) Chrysolaminaritol and laminaritol were each hydrolysed with N-sulphuric acid for 5 hr. at 100°.

(*d*) The methylated polysaccharides (see below) were hydrolysed with 90% formic acid in a sealed tube under carbon dioxide for 4 hr. in a boiling-water bath, then diluted with water (5 vol.) and heated for a further 1 hr. The mixture was filtered from Filter-Cel and glass and the filtrate deionized by passage through a column of mixed-bed resin [Amberlite IR-120(H) and IR-4B(OH)]. The column was washed both with water and with methanol and the combined eluents, after filtration, were evaporated to dryness.

Periodate oxidation. The consumption of periodate and production of formaldehyde were determined by the methods of Aspinall & Ferrier (1957) and Hough, Powell & Woods (1956) respectively. Formaldehyde was also determined with the chromotropic acid reagent by the methods of MacFadyen, Watkins & Anderson (1945), as modified by Parrish (1959) and Hough & Perry (1956). The degree of polymerization was measured from the formaldehyde released on oxidation with periodate. All the experiments with periodate were carried out in duplicate.

Moisture. The loss of weight on drying the chrysolaminarin at 60°/15 mm. mercury over phosphoric oxide for 24 hr. was used to calculate the moisture content.

RESULTS

Isolation and purification of the chrysolaminarin

The material used in these experiments was isolated by Professor H. A. von Stosch by the following procedure.

The water bloom on a small pond in the Botanical Gardens of Darmstadt was collected in early spring. Microscopic examination showed that it contained the following species of diatoms: *Nitzschia sigmoidea*, *Cymatopleura solea*, *Pinnularia* sp. and *Melosira varians*. The mixed culture was extracted with aq. 50% ethanol or hot water, and the extracts were deproteinized by treatment with basic lead acetate, followed by precipitation of the lead with hydrogen sulphide. In some experiments the chrysolaminarin was isolated by allowing it to deposit from the concentrated protein-free solution on standing. In other cases it was crystallized from diacetin and the product washed with ethanol and and ether. Twelve different collections of bloom were made and the crystalline extracts were combined and recrystallized twice from water. Yield was about 1·0 g.

Characterization of chrysolaminarin

The crystalline chrysolaminarin has m.p. 273°, $[\alpha]_D^{18} - 6°$ in water (*c*, 2·0) (moisture, 11%). The dried material has the same crystalline appearance under the microscope and the same m.p. as the initial material. An aqueous solution of the latter gave no colour with iodine solution.

Glucose content. Hydrolysis of the dried chrysolaminarin (50·4 mg.), followed by determination of the reducing value, indicated that the polysaccharide contained 99·5% of glucose, assuming it to be a polymer of glucose. The hydrolysate had $[\alpha]_D^{18} + 52·8°$ in water (*c*, 0·5) and after neutralization with barium carbonate and concentration the resulting syrup crystallized. The crystals had m.p. and mixed m.p. with glucose hydrate 83°. That glucose was the only sugar liberated by acid hydrolysis was shown by incubating a sample of the hydrolysate with notatin, glucose oxidase, at 37° for 6 hr. The resulting solution was non-reducing and chromatographic examination (solvent D, spray iv) showed that the single spot given by the original hydrolysate (solvents A and C, spray i) had been replaced by a spot corresponding to gluconic acid. A hydrolysate derived from laminarin (50 mg.) showed on chromatographic examination (solvent B, spray iii) in addition to glucose a spot identical in speed and colour with mannitol run as a control. This spot was absent from a comparable chromatogram of the hydrolysate from chrysolaminarin.

Partial hydrolysis of laminarin gave a hydrolysate which on chromatographic examination (solvent G, 132 hr. development) revealed spots with R_{Gl} 0·71, 0·56, 0·43, 0·35, 0·26, 0·21, 0·15, 0·13, 0·08. Laminari-biose and -triose run as controls had R_{Gl} 0·71 and 0·43 respectively.

Borohydride reduction. Each of the dried polysaccharides, chrysolaminarin (132·9 mg.) and laminarin (240 mg.), were reduced with potassium borohydride (0·1 g.) in water (10 ml.) with stirring for 48 hr. at room temperature. The mixtures were neutralized (pH 7, acetic acid) and the polysaccharides precipitated with ethanol. They were purified by twice dissolving in water and precipitating with ethanol. Chrysolaminaritol (91% yield) and laminaritol (95%) were isolated with 92% and 93% glucose contents respectively. Insufficient material was available to determine the reducing power of the chrysolaminaritol, but the laminaritol (88 mg.) had no reducing power, and since the reduction of the two polysaccharides was carried out under identical conditions it may be assumed that the reduction of the chrysolaminarin was complete. Reduction with borohydride converts all the reducing glucose residues in laminarin and chrysolaminarin into sorbitol units. The mannitol units are unattacked. Hydrolysates of the two polysaccharide alcohols (30 mg.) were examined by paper chromatography (solvent B, spray iii). From laminaritol spots, corresponding to glucose, mannitol and sorbitol were obtained, and from chrysolaminaritol, glucose and sorbitol. Visual examination indicated a relatively higher concentration of sorbitol from chrysolaminaritol.

Methylation of chrysolaminarin. The dried polysaccharide (85 mg.) was mixed with Filter-Cel

(200 mg.) and methylated in liquid ammonia (about 3 ml.) under less than atmospheric pressure in the micro-apparatus devised by Isbell, Frush, Bruchner, Kowkabany & Wampler (1957). Potassium (in portions of about 3 mg.) was added until the mixture became permanently blue (5 hr.). After complete removal of the ammonia, methyl iodide (2 ml.) was introduced into the reaction vessel and the mixture refluxed with stirring for 18 hr. Then the methyl iodide was withdrawn from the reaction vessel and the methylation repeated five times before isolation of the polysaccharide. The following minor modifications were introduced into the procedure: (1) More efficient condensation of ammonia was achieved when acetone replaced the chlorobenzene in the cold finger. (2) A flat base to the reaction vessel lessened the possibility of the magnetic stirrer sticking. (3) The most satisfactory grease for the glass stopcocks was found to be paraffin and rubber lubricant (British Drug Houses Ltd.). (4) An additional stopcock in a tube at right angles to the side arm from which the potassium was introduced permitted re-evacuation of this arm whenever a fresh supply of potassium was introduced and thus prevented the slight loss of vacuum in the main apparatus. (5) It was found that a hair-drier was suitable for defrosting the stopcock used for the potassium addition, and also for heating the various parts of the glassware to remove completely both the ammonia and the methyl iodide. (6) To ensure easy removal of the cold finger it was found to be advantageous to renew the dry acetone in the outer part of the cold finger after each methylation.

The methoxyl content of the final product was not determined because it proved impossible to free the methylated polymer from contaminating grease.

Examination of the methylated hydrolysates. The partly crystalline solid (70 mg.) obtained on hydrolysis of the methylated chrysolaminarin was examined on a paper chromatogram (solvents E and F, spray i) and by electrophoresis. Spots with speeds identical with 2:3:4:6-tetra-, 2:4:6-tri- and 2:4-di-*O*-methylglucoses were obtained. No trace of 4:6-di-*O*-methylglucose was revealed on ionophoresis (2:4-di-*O*-methyl- and 4:6-di-*O*-methylglucose have M_0 0·09 and 0·20 respectively; Mackie & Percival, 1959). Determination of the relative molar proportions of tetra- to tri- to di-*O*-methylglucose (Schaefer & Van Cleve, 1956) gave 1:10·5:0·46. Some of the crystals were removed from the hydrolysate and freed from adhering syrup. They had m.p. and mixed m.p. with 2:4:6-tri-*O*-methylglucose 118°.

The chromatographic spot corresponding to 2:4:6-tri-*O*-methylglucose was elongated when developed in solvent F and it was suspected that a small proportion of 2:3:4-tri-*O*-methylglucose might be present. The methylated sugars (14·45 mg.) were separated on chromatography paper (solvent E). The tetra-, tri- and di-*O*-methyl sugars were located by means of guide strips. The areas of paper containing the different sugars were cut out and the latter was eluted with water. The quantity of 2:3:4-tri-*O*-methylglucose in the tri-*O*-methyl fraction could not be determined by straightforward oxidation with periodate and determination of the quantity of formaldehyde released since steric factors prevent complete liberation of the latter. Prior reduction to the straight-chain alcohol removes this effect and leads to a quantitative yield of formaldehyde.

The tri-*O*-methyl sugars (5·0 mg.; 9·5 mg.) dissolved in water (2 ml.) were reduced with potassium borohydride (8 mg.). After 2 hr. the solutions were neutralized with sulphuric acid, 0·3 M-sodium periodate (0·3 ml.) was added and the solutions were each made up to 3 ml. and set aside for 30 min. (see Perry & Jones, 1957). A sample (1 ml.) was removed from each and analysed for formaldehyde (MacFadyen *et al.* 1945; Parrish, 1959). 2:4:6-Tri-*O*-methylglucose (2 mg.), 2:4:6-tri-*O*-methylglucitol (2 mg.) and the eluate from a similar area of chromatography paper were oxidized with 0·3 M-sodium periodate as for the reduced tri-*O*-methyl fraction, and the resulting solutions were analysed for formaldehyde. Formaldehyde (57·6 and 114 μg.) was liberated from the solutions (3 ml.) derived from the methylated chrysolaminarin. In contrast no formaldehyde was released in the other three experiments.

Confirmation of the presence of 2:3:4-tri-*O*-methylglucose was obtained by chromatographic examination of the residual periodate oxidation solution derived from methylated chrysolaminarin, after destruction of the excess of periodate with ethylene glycol and deionization. A spot corresponding to 2:3:4-tri-*O*-methylxylose (spray i) was revealed. Reduction of 2:3:4-tri-*O*-methylglucose followed by periodate oxidation yields 2:3:4-tri-*O*-methylxylose.

The yield of formaldehyde from the tri-*O*-methyl fraction from methylated chrysolaminarin corresponds to 1 unit of 2:3:4-tri-*O*-methylglucose to every 11·0 units of tri-*O*-methyl sugar, that is approximately one 1:6′-linked glucose unit to every 11 1:3′-linked residues.

Methylation of laminarin. Laminarin (50 mg.) was methylated twice and then hydrolysed under the conditions used for the chrysolaminarin. Subsequent estimation of the methylated sugars gave tetra-:tri-:di-*O*-methylglucose = 1:16:0·6. Chromatographic separation of the respective sugars and reduction and periodate oxidation of the tri-*O*-methyl (11·2 mg.) fraction released

101 μg. of formaldehyde. This corresponds to about 7% of 2:3:4-tri-*O*-methylglucose and indicates that there is on an average approximately one 1:6′-linked glucose unit to 15 1:3′-linked units.

The presence of one part of di-*O*-methylglucose in about 25 and 19 parts of methylated sugars from chrysolaminarin and laminarin respectively, unless it is due to undermethylation or demethylation or both during hydrolysis, provides evidence for the presence of about one branch point in every molecule. The high proportion of tetra-*O*-methylglucose from both methylated polysaccharides indicates that it is unlikely that either undermethylation or demethylation occurred to any appreciable extent in the present experiments.

Oxidation with periodate. Dried laminarin (14·05 mg.), dried chrysolaminarin (18·59 mg.), laminaritol (19·54 mg.), chrysolaminaritol (16·66 mg.), glucose (15·4 mg.) and sorbitol (24·07 mg.) were each dissolved in 0·015 M-sodium periodate (15 ml.) and stored in the dark at 2°. Samples (1 ml.) were analysed for periodate consumption and for formaldehyde release (Hough, Powell & Woods, 1956). After 48 hr. the consumption of periodate appeared to be complete for both polysaccharides. After oxidation for 96 hr. at 2° the residual oxidation solutions were allowed to stand at room temperature and samples (1 ml.) were analysed as before (see Table 1).

The relatively small quantity of periodate reduced by laminarin provided evidence of chains of 1:3′-linked glucose units (Anderson, Hirst, Manners & Ross, 1958). In the present experiments the similarity in the quantity of periodate consumed by the two polysaccharides indicates a similarity in structure and a high proportion of 1:3′-linked glucose residues in chrysolaminarin, a conclusion which finds support in the presence of a high proportion of unattacked glucose in the hydrolysates of the oxopolysaccharide and in the relatively large quantity of 2:4:6-tri-*O*-methylglucose in the hydrolysate of the methylated polysaccharide. A partial acid hydrolysate of the oxopolysaccharide of chrysolaminarin revealed on chromatographic analysis spots with R_{Gl} 0·71, 0·43, 0·26 and 0·15. The absence of spots with R_{Gl} 0·56 (gentiobiose), 0·35 (possibly 3-*O*-β-gentiobiosylglucose or 1-*O*-β-laminaribiosylmannitol; Peat, Whelan & Lawley, 1958), 0·21, 0·13 given by a hydrolysate of unoxidized laminarin may be explained as due to cleavage of all the 1:6′-linked units in chrysolaminarin by the periodate and consequently the loss of all the 1:6′-linked oligosaccharides.

The action of periodate on laminarin under various conditions is described in detail by Anderson *et al.* (1958), and these and other workers (Peat *et al.* 1958) consider that laminarin, like starch, is made up of two molecular species. In one of these the chains of 1:3′-linked glucose units are terminated by reducing glucose residues (G-chains) and in the other these residues are replaced by mannitol units (M-chains) linked through $C_{(1)}$ or $C_{(6)}$. More recently Smith & Unrau (1959) have provided evidence that each mannitol unit in the mannitol-containing molecules is linked through $C_{(1)}$ and $C_{(2)}$ (or $C_{(6)}$ and $C_{(5)}$) to two glucose chains and that these molecules contain two 1:6′-linked glucose units, whereas the molecules devoid of mannitol contain only one 1:6′-linkage.

When subjected to oxidation with periodate the residues in laminarin that are attacked are the 1:6′-linked units and the residues at the non-reducing ends of the chains, each of which reduce

Table 1. *Oxidation of the polysaccharides with periodate*

Oxidation was carried out with 15 mM-sodium periodate in the dark. After 96 hr. at 2° the oxidation was continued at room temperature (18°). Other details are given in the text.

(*a*) $NaIO_4$ consumed (mole/anhydroglucose unit)

	Oxidation at 2°		Subsequent oxidation at 18°
	48 hr.	96 hr.	(6 days)
Laminarin	0·221	0·221	0·364
Chrysolaminarin	0·291	0·309	0·445

(*b*) Formaldehyde produced (mole/anhydroglucose unit)

	Oxidation at 2°			Subsequent oxidation at 18°			
	24 hr.	48 hr.	96 hr.	96 hr.	5 days	6 days	7 days
Laminarin	0·028	0·028	0·024	—	0·0468	0·0469	0·0469
Chrysolaminarin	0·009	0·008	—	—	0·0462	0·0470	0·0470
Glucose	0·009	0·009	—	—	—	—	—
Laminaritol	—	0·056	0·056	0·066	—	0·069	—
Chrysolaminaritol	—	0·060	0·056	0·077	—	0·080	—
Sorbitol	—	—	0·77	—	—	0·91	—

2 moles of periodate with the release of 1 mole of formic acid. At the same time the units at the other ends of the chains are also cleaved. If the reaction is carried out at 2° the oxidation of these latter units in the G-chains is arrested after the reduction of 1 molecule of periodate owing to the formation of a formyl ester, which is hydrolysed very slowly at this temperature, and no formaldehyde is formed. The mannitol units at this end of the chains release 1 mole of formaldehyde (Anderson *et al.* 1958).

The release of 0·028 mole of formaldehyde for each anhydroglucose unit in laminarin corresponds to 2% of mannitol present in laminarin and is in agreement with the results of the earlier workers. The value recorded for chrysolaminarin (Table 1) is no greater than that for glucose, which is known to yield no formaldehyde under these conditions. This confirms the absence of M-chains in this polysaccharide.

At room temperature both M-chains and G-chains release 1 mole of formaldehyde and from this release the average degree of polymerization of the polysaccharide may be calculated. In the present experiments (see Table 1) 1 mole of formaldehyde is released by every 21 anhydroglucose units in both polysaccharides.

The reduced chrysolaminarin should release 2 moles of formaldehyde from each of the newly formed sorbitol units and, assuming the absence of M-chains, chrysolaminaritol should give about 30% more formaldehyde than laminaritol. In fact, the yield of formaldehyde on oxidation of chrysolaminaritol at room temperature almost reaches the expected figure: 0·08 mole/anhydroglucose unit instead of the theoretical 0·090 mole for the complete absence of mannitol-ended chains. This yield is considerably higher than that from laminaritol and is further evidence that M-chains, if present in chrysolaminarin, must be very small in number. Comparable experiments with sorbitol show that 100% release of formaldehyde on oxidation with periodate is very difficult to attain and this may explain the slightly low yield from chrysolaminaritol. It will be observed from Table 1 that the yield of formaldehyde at 2° in 96 hr. is low, but this can be explained by the observed slow release from sorbitol at this temperature.

Oxidation with excess of periodate at pH 8 *and* 35°. Portions (1 ml.) of each of the polysaccharide solutions prepared as described in the previous section were withdrawn immediately after mixing, and phosphate buffer (6·8 ml., pH 8), together with excess of sodium periodate (1 ml., 0·3 M), were added and the solutions diluted to 10 ml. with water. After incubation at 35° the release of formaldehyde was determined on samples (1 ml.) (Hough & Perry, 1956) and found to be complete after 48 hr. The amounts of formaldehyde released (mole/anhydroglucose unit) were: laminarin, 0·600; laminaritol, 0·603; chrysolaminarin, 0·610; chrysolaminaritol, 0·563.

It has been observed (Anderson *et al.* 1958) that oxidation of this type of polysaccharide under these conditions results in the stepwise removal of glucose and sorbitol units from the ends of the chains originally carrying reducing groups, through the formation of an intermediate malonaldehyde grouping, and 1 mole of formaldehyde is liberated from each glucose residue that is degraded. Mannitol residues linked through $C_{(1)}$ or $C_{(6)}$ and glucose units linked through $C_{(6)}$ do not overoxidize, as no malonaldehyde grouping is formed. The presence of 40% of laminarin and laminaritol which is resistant to overoxidation is in agreement with the structure advanced by earlier workers.

In the absence of M-chains the incomplete oxidation of chrysolaminarin and its alcohol confirms the presence in the molecule of 1:6′-linked glucose units or branch points (or both) which appear to occur on an average just over half way along the chains.

DISCUSSION

Results of these investigations agree with the earlier findings on laminarin that this polysaccharide consists essentially of chains of β-1:3′-linked glucose units and that some of the chains are terminated at the reducing end by mannitol residues. The results provide the first definite evidence for the presence of 2:3:4-tri-*O*-methylglucose in the methylated hydrolysate and hence confirm the presence of 1:6′-linkages in a linear chain of β-1:3′-linked glucose residues. This is in accordance with the interpretation of Peat *et al.* (1958) from partial-hydrolysis studies on laminarin, and of Smith & Unrau (1959) from their periodate-oxidation and -reduction studies on this polysaccharide. Although the present experiments indicate that on an average 6% of the units in the linear chains are 1:6′-linked they provide no evidence for the relative position of the 1:3′- and 1:6′-linkages in the molecule. The balance of evidence from previous work in these laboratories (Anderson *et al.* 1958; Hirst, O'Donnell & Percival, 1958) was in favour of the occurrence of 1:6′-linked glucose units only at branch points in the laminarin molecule and the present methylation studies suggest that on an average one branch point at $C_{(6)}$ is present in each molecule. It follows that 1:6′-linkages may be present in laminarin both in the linear chains and as interchain linkages. Further work is in progress to substantiate this. The proportion of tetra-*O*-methylglucose agrees with the chain length of 16 glucose residues recorded by Barry (1942) from

successive oxidation with periodate and bromine. The lower proportion of tetra-*O*-methylglucose reported by other workers was not from whole laminarin but from fractions of higher molecular weight.

It should be mentioned that the present results differ from those of Smith & Unrau (1959) in that no evidence could be obtained for the presence of mannose in the sample of laminarin under investigation.

Chrysolaminarin, the polysaccharide synthesized by diatoms, has been shown to resemble laminarin in many respects. It consists of β-1:3′- and 1:6′-linked glucose units in the ratio of 11:1 (cf. laminarin 15:1) with an average of one branch point at $C_{(6)}$ in every molecule. However, the average chain length, calculated from the proportion of tetra-*O*-methylglucose present in the methylated hydrolysate, appears to be smaller in the chrysolaminarin: 12 glucose units instead of 16 units calculated for laminarin. Furthermore, whereas it was confirmed that a proportion of the laminarin molecules corresponding to 2% of mannitol are terminated at the reducing end by this sugar alcohol, no evidence for the presence of any unit other than glucose at the reducing end of the chrysolaminarin molecules could be obtained. This polysaccharide appears to differ from laminarin therefore in that it comprises only one of the types of molecule (G-chains) found in the latter.

From the formaldehyde liberated on oxidation with periodate both the chrysolaminarin and the sample of laminarin investigated appear to have a molecular weight of about 3500. This agrees with the figure reported by Broatch (1956) for methylated laminaritol.

SUMMARY

1. Crystalline chrysolaminarin separated from a mixture of diatoms (Chrysophyceae) had m.p. 273°, $[\alpha]_D - 6°$ and was shown to contain 99·5% of glucose. Chromatographic examination of total hydrolysates of laminarin and of chrysolaminarin revealed the presence of mannitol in the former but not in the latter.

2. The alcohols obtained on reduction of the polysaccharides with borohydride gave on hydrolysis glucose, sorbitol and mannitol from laminarin and glucose and sorbitol from chrysolaminarin.

3. 2:3:4:6-Tetra-*O*-methylglucose, tri-*O*-methylglucose and di-*O*-methylglucose in the ratio of 1:10·5:0·46 and 1:16:0·6 were present in the hydrolysates of methylated chrysolaminarin and laminarin respectively. The presence of the di-*O*-methyl sugar indicates the possible presence of one branch point per molecule.

4. The methylated sugars were separated on thick paper and evidence was obtained that both tri-*O*-methyl fractions consisted of a mixture of 2:4:6- and 2:3:4-tri-*O*-methylglucoses, in the ratio of about 11:1 and 15:1 in chrysolaminarin and laminarin respectively. This provides proof of the presence of 1:6′-linkages in the linear 1:3′-linked chains.

5. The amount of periodate reduced by both polysaccharides is in agreement with the presence of a high proportion of 1:3′-linked glucose units.

6. The quantity of formaldehyde released by laminarin and chrysolaminarin and their respective alcohols on oxidation with periodate confirms the presence of mannitol-terminated chains (M-chains) in laminarin. No evidence of M-chains in chrysolaminarin could be obtained.

7. The degree of polymerization of both polysaccharides calculated from formaldehyde release is about 21 glucose units.

8. The incomplete oxidation of the two polysaccharides under conditions which facilitate degradation of 1:3′-linked glucose chains provides confirmation of the presence of 1:6′-linked units in chrysolaminarin and of similar linkages or M-chains or both in laminarin.

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The Effect of Succinate and Amytal on the Reduction of Acetoacetate in Animal Tissues

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It has recently been shown (Kulka, Krebs & Eggleston, 1961) that succinate promotes the formation of β-hydroxybutyrate from acetoacetate in sheep-heart homogenates. An explanation was put forward which is based on the fact that succinate is oxidized in preference to substrates requiring a pyridine nucleotide-linked dehydrogenase. As the electrons from succinate and from reduced pyridine nucleotide travel to oxygen by a common pathway at the cytochrome level the preferential availability of the electrons from succinate blocks the transfer of electrons from reduced pyridine nucleotide to oxygen. At the same time the oxidation of succinate provides a substrate, malate, which reduces pyridine nucleotide. These two circumstances increase the amounts of reduced pyridine nucleotide available for the reduction of acetoacetate. This paper presents further evidence in support of this concept.

METHODS

Materials. Sodium acetoacetate and DL-β-hydroxybutyrate samples were as previously described. Crystalline L-lactic acid was prepared according to Krebs (1960), α-oxoglutaric acid according to Friedman & Kosower (1955); succinate-free crystalline monosodium oxoglutarate was prepared as follows. The crude acid was dissolved in 3 vol. of water and the solution was adjusted to pH 3·7 with solid Na_2CO_3, pH being checked with a pH meter. The adjustment to this pH is critical as pK_1 was found to be 2·47, pK_2 4·68 (determined electrometrically at 22°). After filtration 1 vol. of ethanol was added slowly and the resultant turbid solution was kept overnight in the refrigerator. The precipitate formed contained mostly impurities. A further volume of ethanol was added to the supernatant and after storage for 24 hr. at 0° crystals of monosodium α-oxoglutarate were collected. The purity was 96·5%. A third volume of ethanol yielded material of 100% purity. This was used for the experiments. Amytal (5-ethyl-5-*iso*amylbarbiturate) was added as the sodium salt.

Tissue preparations. Tissue homogenates were prepared and handled as previously described (Kulka *et al.* 1961). Any modifications in incubation time and tissue concentration are stated in the description of the experiments. In most cases the enzymic reactions were stopped by the addition of 1 ml. of N-HCl/4 ml. of homogenate. In other cases HCl was replaced by 15% perchloric acid.

Analytical methods. Acetoacetate was determined according to Edson (1935) or Walker (1954), citrate according to Taylor (1953), succinate according to Rodgers (1961), the sum of malate and fumarate according to Nossal (1952), pyruvate and α-oxoglutarate according to Holzer & Holldorf (1957), malate and lactate according to Hohorst, Kreutz & Bücher (1959), glutamate according to Krebs & Bellamy (1960), β-hydroxybutyrate according to

Table 1. *Yield of 'β-hydroxybutyrate' of various compounds treated according to Greenberg & Lester* (1944)

The quantities tested correspond to maximum amounts present in the homogenates analysed.

Substance tested (16 μmoles)	'β-Hydroxybutyrate' found (μmole)
Succinate	0·05
Fumarate	0
L-Malate	0
Citrate	0·25
α-Oxoglutarate	0·16
Pyruvate	0·36
L-Lactate	0·05
Butyrate	0·05
Crotonate	0·14
Glucose	0·09
Amytal (5 μmoles)	0·08

Table 2. *Effect of succinate on the aerobic reduction of acetoacetate in various tissues*

Each vessel contained 4 ml. of medium, including 20 μmoles of acetoacetate. Incubated for 45 min. at 30°; gas space, O_2. The dry weight of the epididymal fat body refers to fat-free tissue.

Tissue	Type of material	Amount of dry tissue (mg./4 ml.)	Concn. of succinate (mM)	Uptake of O_2 (μmoles)	Acetoacetate removed (μmoles)	β-Hydroxybutyrate formed (μmoles)	'Acetoacetate oxidized' (μmoles)	O_2-equivalents used / acetoacetate oxidized
Liver (rat)	Homogenate	120	0	30·0	0·4	0·88	0	—
		120	20	59·1	10·9	10·0	0·9	71
Testis (rat)	Teased-out tissue	160	0	33·2	5·2	0·4	4·8	7·0
		150	20	58·4	7·8	2·64	5·2	11·5
Epididymal fat body (rat)	Whole	28	0	9·9	2·1	0·20	1·9	5·3
		29	20	12·4	2·3	1·23	1·1	11·8
Diaphragm (sheep)	Homogenate	150	0	11·2	12·9	9·88	3·0	5·4
		150	20	30·3	16·4	13·9	2·5	15·0
Diaphragm (cattle)	Homogenate	150	0	9·5	4·9	3·13	1·8	6·2
		150	20	21·1	5·3	4·69	0·6	39
Diaphragm (rat)	Homogenate	120	0	14·3	6·1	1·66	4·4	3·1
		120	20	22·8	6·3	2·44	3·9	6·2
Brain (rat)	Homogenate	90	0	5·4	1·6	0·38	1·2	4·7
		90	20	24·0	2·6	0·63	2·0	12·1
Muscle, neck (sheep)	Homogenate	180	0	6·4	4·2	4·1	~ 0	—
		180	20	15·8	5·3	5·8	~ 0	—
Muscle, leg (rat)	Homogenate	150	0	17·0	1·5	0	1·5	11·3
		150	20	32·4	2·5	0·5	2·0	16·4
Muscle, breast (pigeon)	Latapie-mince	150	0	62·0	6·8	0	6·8	9·1
		150	20	102·5	10·5	1·20	9·3	9·1
Kidney cortex (sheep)	Homogenate	180	0	60·0	14·6	1·7	12·9	4·7
		180	20	90·0	18·8	7·7	11·1	8·5
Kidney cortex (sheep)	Homogenate	120	0	21·8	5·8	2·9	2·9	8·1
		120	20	49·9	10·1	3·7	6·4	8·1

Greenberg & Lester (1944) as described by Kulka *et al.* (1961). This method, based on the oxidation of β-hydroxybutyrate to acetone by chromate, is not absolutely specific but interference by added substrates or tissue constituents was negligible. Tests of the reactivity of the added substrates gave the values shown in Table 1. They were very low compared with those of the β-hydroxybutyrate arising in the presence of acetoacetate. The method may therefore be regarded as adequate.

Comments on the interpretation of results. Any disappearance of acetoacetate which was not accounted for by the appearance of β-hydroxybutyrate was taken to represent an oxidation of acetoacetate, and if no intermediates of the tricarboxylic acid cycle accumulated the oxidation was assumed to be complete. Reactions other than oxidation and reduction no doubt occurred, such as the decarboxylation to acetone and the synthesis of cholesterol, but the rates of these reactions were probably negligible under the test conditions. This is suggested by the observation that in liver, where the rates of the side reactions are expected to be higher than in other tissues, virtually all acetoacetate was recovered as β-hydroxybutyrate in many experiments.

In order to calculate the contribution which the oxidation of acetoacetate makes to respiration it is necessary to know the amounts of hydrogen acceptors available for the oxidation of acetoacetate. Whilst O_2 is as a rule the only major hydrogen acceptor in animal tissues this is not the case in the presence of acetoacetate, which can act as an additional hydrogen acceptor. In this case the amounts of available hydrogen acceptors are therefore the sum of the O_2 uptake and the β-hydroxybutyrate formation. In subsequent calculations this sum has been expressed as 'O_2-equivalent', 2 moles of β-hydroxybutyrate were equated to 1 mole of O_2 and added to the O_2 uptake.

RESULTS

Effect of succinate on the reduction of acetoacetate in various tissues

The effect of succinate previously described for sheep-heart muscle was also found, in varying degrees, in all other tissues tested (Table 2). Quantitatively the effect of succinate was greatest in liver, where in the example quoted the addition of succinate increased the rate of β-hydroxybutyrate formation tenfold. In this tissue almost the whole of the acetoacetate removed aerobically in the presence of succinate was accounted for by the formation of β-hydroxybutyrate. In other tissues the amounts of acetoacetate removed were greater than those of β-hydroxybutyrate formed; the excess presumably underwent oxidation.

Acetoacetate as a fuel of respiration

In sheep-heart homogenates the ratio of O_2-equivalents to acetoacetate oxidized was in many cases near 4 (Kulka *et al.* 1961). If no intermediates accumulate and if the loss of acetoacetate by decarboxylation to acetone is negligible (as was the case under the conditions of the present experiments) a value of 4 indicates that the whole respiration is due to the oxidation of acetoacetate, and if the value is n (where n should be greater than 4) the percentage respiration due to the oxidation of acetoacetate is $100 \times 4/n$.

According to the data in the last column of Table 2 acetoacetate, when added as sole substrate, contributed substantially, i.e. more than 50%, to the respiration of the diaphragms of the rat, sheep and cattle and of epididymal fat. It also contributed much to the respiration of testis and brain but in these tissues the respiration was rather lower than in the intact tissue owing to damage caused by homogenization or to the absence of glucose or both. No oxidation of acetoacetate occurred in liver. Succinate decreased in most tissues the proportion contributed to respiration by acetoacetate and also decreased the absolute amount of acetoacetate oxidized.

Effect of succinate concentration on the aerobic reduction of acetoacetate

Small quantities of succinate (1 μmole/4 ml.) had no appreciable effect on the formation of β-hydroxybutyrate. Relatively large quantities of

Table 3. *Effect of succinate concentration on the aerobic reduction of acetoacetate in sheep-heart and rat-liver homogenate*

Incubated for 45 min. at 30°; gas space, O_2. Data refer to 4 ml. of 10% homogenate. Expt. 1, sheep heart; Expts. 2 and 3, rat liver.

Expt.	Substrates added	Uptake of O_2 (μmoles)	Acetoacetate removed (μmoles)	β-Hydroxybutyrate formed (μmoles)
1	None	19·8	—	0·4
	Acetoacetate (23 μmoles)	34·3	9·5	4·0
	Acetoacetate (23 μmoles); succinate (1 μmole)	34·5	8·9	4·6
	Acetoacetate (23 μmoles); succinate (10 μmoles)	38·0	11·1	6·1
	Acetoacetate (23 μmoles); succinate (80 μmoles)	65·5	11·8	9·1
2	Acetoacetate (18·7 μmoles)	30·0	1·9	1·5
	Acetoacetate (18·7 μmoles); succinate (10 μmoles)	38·1	8·7	8·9
	Acetoacetate (18·7 μmoles); succinate (80 μmoles)	54·3	13·9	13·7
3	Acetoacetate (20·4 μmoles)	25·1	0	2·2
	Acetoacetate (20·4 μmoles); succinate (1 μmole)	30·5	0·6	1·7
	Acetoacetate (20·4 μmoles); succinate (2 μmoles)	33·2	1·5	3·2

succinate (80 μmoles/4 ml.) were required for maximum effects (Table 3). There was no simple stoicheiometric relation between the amounts of succinate added and extra β-hydroxybutyrate formed but in all experiments less than 1 molecule of β-hydroxybutyrate was formed per molecule of succinate added. These observations are in accordance with the assumption that the stimulation of β-hydroxybutyrate formation by succinate is due to the blockage of electron transport from reduced diphosphopyridine nucleotide ($DPNH_2$) to O_2. No stoicheiometry is expected because only a continuous oxidation of succinate can bring about an effective blocking.

Preferential oxidation of succinate (general considerations)

Data shown in Table 2 already indicate that added succinate is oxidized in preference to acetoacetate. This follows from the fact that succinate increased the rate of oxygen consumption in all tissues, whereas it did not increase or even decrease the amounts of acetoacetate oxidized. The preferential oxidation of succinate also expresses itself in a rise of the value of the ratio O_2-equivalents used/acetoacetate oxidized. The high value of this ratio in the presence of succinate indicates that substances other than acetoacetate served as substitutes of respiration.

In order to examine in more detail the nature of the oxidative reactions in the presence of succinate further experiments were carried out on the following basis. When an intact tissue or a tissue homogenate respires, many oxidizable substrates, endogenous or added, may be available. In the complex system information on the nature of substrate changes can be obtained from simultaneous measurements of the uptake of O_2 and the changes in the concentrations of the starting materials and intermediary metabolites. The chief intermediates which accumulate under most conditions are those of the tricarboxylic cycle, and of the reactions closely related to it. The following substances were therefore determined: succinate, citrate, α-oxoglutarate, pyruvate, lactate, acetoacetate, β-hydroxybutyrate, acetate and the sum of fumarate and malate. The last-named were not measured individually because they always occur together in animal tissues and have the same oxidation level; the value of the sum of their concentration is therefore sufficient for many purposes. *iso*Citrate and *cis*-aconitate are not easily determined at physiological concentrations but in the presence of aconitase their concentrations may be taken to be 11% of that of citrate (Eggleston & Krebs, 1949), and this amount was added to the observed citrate value so as to include all three tricarboxylic acids. Oxaloacetate was not determined. Unless special precautions are taken it is rapidly decarboxylated to pyruvate, and in the present experiments it was included in the pyruvate determination.

In order to calculate the nature of the substrate changes from the changes in the amounts of the intermediary metabolites it is necessary to make some arbitrary assumptions. It was assumed that the intermediates found were all derived from succinate when this was added, rather than from endogenous material or the added acetoacetate,

Table 4. *Relations between formation of intermediates and consumption of oxygen and succinate*

The assumption is made that succinate is the sole precursor of the intermediates.

Intermediates formed	Required for the formation of metabolite from succinate: O_2 (μmoles)	Succinate (μmoles)
Fumarate or malate (1 mole)	0·5	1
Pyruvate (1 mole)	1	1
Citrate (1 mole)	2·5	2
α-Oxoglutarate (1 mole)	3	2
Lactate (1 mole)	0·5	1

Table 5. *Competition of succinate with other substrates in sheep-heart homogenate*

Incubated for 60 min. at 30°; gas space, O_2. Data refer to 8 ml. of 10% homogenate.

Vessel no.	1	2	3	4
Substrates added	None	Acetoacetate (40 μmoles)	Acetoacetate (40 μmoles) and succinate (160 μmoles)	Succinate (160 μmoles)
	Metabolic changes (μmoles)			
O_2	−24·4	−53·2	−108·8	−109
Acetoacetate	0	−17·0	− 17·0	0
β-Hydroxybutyrate	+ 0·6	+ 7·8	+ 18·6	+ 1·6
Succinate	0	0	−159·0	−143·0
Fumarate + malate	0	0	+112·4	+108·0
α-Oxoglutarate	0	0	+ 6·4	+ 3·2
Citrate + *iso*citrate + *cis*-aconitate	+0·32	+ 1·0	+ 10·8	+ 4·4

and on this basis the relations between the formation of intermediates and the consumption of O_2 and succinate are as shown in Table 4.

Succinate and acetoacetate in sheep heart and rat livers

When acetoacetate was added to sheep-heart homogenate as the sole substrate a major part of the respiration was due to an oxidation of acetoacetate (Table 5). The amount of acetoacetate oxidized was 9·2 μmoles and as no appreciable quantities of intermediates were found the acetoacetate was presumably completely oxidized. This accounts for 36·8 μmoles of O_2 or 65% of the O_2-equivalents used (53·2 μmoles of O_2 and 3·9 O_2-equivalents of β-hydroxybutyrate). In contrast there was no acetoacetate oxidized in the presence of succinate: the acetoacetate which had been removed all appeared as β-hydroxybutyrate. From the amounts of fumarate, malate, α-oxoglutarate and citrate formed it follows that the formation of these intermediates accounted for about 90% of

Table 6. *Analysis of the oxidative reactions in vessels* 3 *and* 4 *of Table* 5 (*sheep heart*)

	Vessel 3 (acetoacetate and succinate added)		Vessel 4 (succinate added)	
Metabolite formed	O_2 required (μmoles)	Succinate required (μmoles)	O_2 required (μmoles)	Succinate required (μmoles)
Fumarate and malate	56·2	112·4	54·0	108·0
Citrate, *iso*citrate, *cis*-aconitate	27·0	21·6	11·0	8·8
α-Oxoglutarate	19·2	12·8	9·6	6·4
Sum of all metabolites	102·4	146·8	74·6	123·2
O_2 not accounted for	6·4	—	34·4	—
O_2-equivalents (including β-hydroxybutyrate) not accounted for	15·8	—	35·2	—
Succinate not accounted for	—	12·2	—	26·2

Table 7. *Competition of succinate with other substrates in rat-liver homogenate*

Incubated for 60 min. at 30°; gas space, O_2. Data refer to 8 ml. of 10% homogenate.

	Metabolic changes (μmoles)			
Vessel no.	1	2	3	4
Substrates added	None	Acetoacetate (40 μmoles)	Acetoacetate (40 μmoles) and succinate (160 μmoles)	Succinate (160 μmoles)
O_2	−40·7	−40·4	− 79·4	− 79·8
Acetoacetate	0	− 1·1	− 16·3	0
β-Hydroxybutyrate	0	+ 1·0	+ 14·8	+ 0·4
Succinate	0	0	−139·0	−130·2
Fumarate + malate	0	0	+106·4	+117·2
α-Oxoglutarate	+ 0·42	+ 0·26	+ 0·53	+ 0·81
Citrate + *iso*citrate + *cis*-aconitate	+ 0·37	+ 1·78	+ 4·79	+ 1·70
Pyruvate	− 0·22	+ 0·22	+ 2·78	+ 2·43
Lactate	− 0·34	− 2·76	+ 0·89	− 0·36

Table 8. *Analysis of the oxidative reactions in vessels* 3 *and* 4 *of Table* 7 (*rat liver*)

	Vessel 3 (acetoacetate and succinate added)		Vessel 4 (succinate added)	
Metabolite formed	O_2 required (μmoles)	Succinate required (μmoles)	O_2 required (μmoles)	Succinate required (μmoles)
Fumarate and malate	53·2	106·4	58·6	117·2
Citrate, *iso*citrate, *cis*-aconitate	13·6	11·6	4·7	3·7
α-Oxoglutarate	1·5	1·1	2·4	1·6
Pyruvate	2·8	2·8	2·4	2·4
Lactate	1·8	3·6	0	0
Sum of all metabolites	72·9	125·5	68·1	124·9
O_2 not accounted for	6·5	—	11·7	—
O_2-equivalents (including β-hydroxybutyrate) not accounted for	13·9	—	11·9	—
Succinate not accounted for	—	13·5	—	5·3

the oxygen consumption and 90 % of the succinate removal (Table 6). Thus on addition of succinate plus acetoacetate, or of succinate alone, the oxidation of succinate constituted the main oxidative process, i.e. succinate largely suppressed the oxidation of endogenous material as well as of the added acetoacetate.

In rat liver the action of succinate had similar effects (Table 7). As acetoacetate is not appreciably oxidized in this tissue, its only major reaction under the test conditions is the reduction to β-hydroxybutyrate. In the presence of succinate 80–90 % of the oxidation was accounted for by the formation of the intermediates which were determined (Table 8).

There is no definite information on the nature of the reactions of O_2 and succinate not accounted for by the products measured in the two tissues. This fraction is relatively small; it was calculated as the difference of many individual determinations and therefore a considerable experimental error cannot be excluded. Some succinate or endogenous substrate may have undergone complete oxidation. It is also possible that small quantities of other metabolites that were not determined were formed from succinate.

Effects of substances other than succinate on the reduction of acetoacetate

Among the substances tested in the previous investigation succinate was the only substance which in sheep heart promoted the reduction of acetoacetate aerobically but not anaerobically. As the effect of succinate was much more pronounced in liver than in heart the effect of further substances on the reduction of acetoacetate was examined in liver homogenates. Anaerobically there were no effects on additions of succinate, acetate, propionate, butyrate, crotonate and glucose. Lactate had a slight effect (Table 9); large accelerations of the anaerobic reduction were caused by fumarate, citrate, *iso*citrate, glutamate, α-oxoglutarate and pyruvate. The yield of β-hydroxybutyrate was in most cases somewhat greater than the amount of acetoacetate removed, especially in the presence of pyruvate, and even the

Table 9. *Effects of various substrates on the aerobic and anaerobic removal of acetoacetate in rat liver*

Data refer to 4 ml. of 10 % homogenate. Acetoacetate (20 μmoles) was added to 4 ml. and 80 μmoles of all other substrates. Incubated for 45 min. at 30°; gas space, O_2.

		Anaerobic changes		Aerobic changes		
Expt.	Substrate added other than acetoacetate	Acetoacetate (μmoles)	β-Hydroxybutyrate (μmoles)	O_2 (μmoles)	Acetoacetate (μmoles)	β-Hydroxybutyrate (μmoles)
1	None	− 5·85	+ 6·8	−27·6	− 1·2	+ 2·2
	α-Oxoglutarate	− 8·55	+10·6	−31·1	−11·7	+14·3
	L-Glutamate	−11·9	+13·3	−38·5	−12·6	+15·9
	Pyruvate	− 7·70	+15·2	−34·5	− 0·8	+ 5·9
	L-Lactate	− 7·20	+ 8·9	−35·2	− 0·5	+ 2·1
2	None	− 4·38	+ 6·25	−22·8	− 1·4	+ 2·9
	Fumarate	−10·0	+10·7	−33·2	− 5·2	+ 3·5
	α-Oxoglutarate	− 9·83	+11·2	−30·1	− 8·3	+ 8·7
	Fumarate + α-oxoglutarate	−10·1	+11·5	−28·4	− 6·0	+ 7·4
3	None	− 4·65	+ 5·9	−24·9	− 0·2	+ 2·0
	Succinate	− 3·45	+ 5·4	−57·4	− 8·9	+12·3
	α-Oxoglutarate	− 9·85	+11·6	−33·4	− 8·7	+11·0
	L-Glutamate	−11·3	+12·4	−37·8	−12·8	+16·3
4	None	− 5·2	+ 7·1	−26·7	− 1·4	+ 3·4
	Fumarate	−11·4	+15·5	−33·6	− 5·4	+ 7·9
	Citrate	−20·0*	+23·6	−28·8	− 8·5	+13·4
	L-Glutamate	−16·4	+19·3	−37·4	−15·0	+20·1
5	None	− 5·95	+ 6·4	−26·9	− 0·5	+ 1·0
	Citrate	−18·3	+20·4	−26·5	− 7·0	+11·6
	Glucose	− 6·1	+ 7·3	−26·7	+ 0·4	+ 1·6
	Pyruvate (no acetoacetate)	+ 0·45	+ 4·1	−34·5	+ 4·9	+ 3·8
6	None	− 5·2	+ 5·38	−24·2	− 0·3	+ 0·8
	Fumarate	− 9·9	+10·7	−34·5	− 4·15	+ 4·9
	L-Malate	− 9·8	+11·7	−31·0	− 8·1	+ 9·1
	Oxaloacetate	− 1·9	+ 2·37	−16·3	− 4·7	+ 5·4
7†	None	− 4·0	+ 5·0	—	—	—
	Citrate	−13·2	+14·0	—	—	—
	*iso*Citrate	−16·6	+17·9	—	—	—

* All acetoacetate added was removed.

† In Expt. 7, 40 μmoles of citrate were added and the incubation time was 20 min.

Table 10. *Effects of 2:4-dinitrophenol on the removal of acetoacetate and the formation of β-hydroxybutyrate in rat-liver homogenate*

Incubated at 30°; gas space, O_2. Data refer to 4 ml. of 10% homogenate. All suspensions contained 20 μmoles of acetoacetate. 2:4-Dinitrophenol was added with substrate from the side arm at zero time.

Expt.	Further substrates added (μmoles)	Dinitrophenol (μM)	Period of incubation (min.)	Uptake of O_2 (μmoles)	Acetoacetate removed (μmoles)	β-Hydroxybutyrate formed (μmoles)
1	None	0	20	11·7	0·4	0·43
	Succinate (80 μmoles)	0		28·5	10·2	10·7
	None	50		5·7	1·3	0·88
	Succinate (80 μmoles)	50		42·3	3·8	3·44
2	None	0	20	16·1	0·5	1·50
	Succinate (80 μmoles) and pyruvate (20 μmoles)	0		31·3	5·9	8·00
	α-Oxoglutarate (80 μmoles)	0		14·8	4·4	5·25
	None	50		6·5	0·6	2·58
	Succinate (80 μmoles) and pyruvate (20 μmoles)	50		44·5	3·9	4·97
	α-Oxoglutarate	50		31·7	0·7	1·25
3	None	0	30	17·6	2·1	1·50
	Succinate (80 μmoles)	0		39·8	10·9	11·4
	None	10		18·3	1·8	1·49
	Succinate (80 μmoles)	10		44·9	9·6	9·12
4	None	0	20	11·7	0·7	0·25
	Succinate	0		27·8	12·4	13·4
	None	25		8·1	0·8	0·88
	Succinate	25		40·2	7·4	6·8
	None	50		7·1	1·1	0·5
	Succinate	50		49·0	2·6	2·3
	None	100		6·8	0·5	0·13
	Succinate	100		41·8	1·6	0·88

Table 11. *Effect of amytal on metabolism of acetoacetate*

Data refer to 4 ml. of 10% homogenate. Incubated for 45 min. at 30°; gas space, O_2. Acetoacetate added: 20 μmoles. Succinate added: 80 μmoles.

Tissue	Substrates added	Sodium amytal (final concn.) (mM)	Uptake of O_2 (μmoles)	Acetoacetate (μmoles)	β-Hydroxybutyrate (μmoles)
Rat liver	Acetoacetate	0	−27·3	− 2·2	+ 1·3
	Acetoacetate; succinate	0	−56·5	−11·8	+11·6
	Acetoacetate	1	−11·7	− 7·1	+ 6·1
	Acetoacetate; succinate	1	−53·8	−14·6	+15·7
	Acetoacetate	2	−10·3	− 6·3	+ 5·8
	Acetoacetate; succinate	2	−54·8	−16·6	+17·2
Sheep heart	Acetoacetate	0	−27·2	−10·9	+ 4·3
	Acetoacetate; succinate	0	−56·0	−15·1	+11·4
	Acetoacetate	1	−21·0	−13·4	+ 8·3
	Acetoacetate; succinate	1	−43·0	−16·8	+17·3
Sheep heart	Acetoacetate	0	−21·0	−10·6	+ 2·5
		1·25	− 8·3	− 8·3	+ 5·4
		2·5	− 4·33	−10·5	+10·0
		5·0	− 3·12	− 9·4	+ 8·25
	Acetoacetate; succinate	0	−46·0	−12·7	+ 9·25
		1·25	−41·5	−10·9	+10·0
		2·5	−35·2	−13·7	+13·5
		5·0	−36·4	−12·5	+13·3

homogenate to which no acetoacetate had been added formed small amounts of β-hydroxybutyrate. Whilst the initial β-hydroxybutyrate value could have been due to the lack of specificity of the analytical method this is unlikely to apply to the additional β-hydroxybutyrate formed on incubation. Presumably acetyl-coenzyme A formed anaerobically, especially from pyruvate (see Expts. 1 and 5), condensed to yield acetoacetate which was reduced by the $DPNH_2$ formed in the conversion of pyruvate or other substrates into acetyl-coenzyme A. The most rapid anaerobic formation of β-hydroxybutyrate occurred on addition of citrate and of glutamate. The effects of α-oxoglutarate and fumarate were not additive.

Succinate was again the only substance which strongly stimulated the formation of β-hydroxybutyrate under aerobic conditions but had no effect anaerobically. In some experiments α-oxoglutarate and glutamate generated more β-hydroxybutyrate aerobically than anaerobically but the differences were relatively small and can be accounted for by the formation of succinate from these two substrates.

Effects of 2:4-*dinitrophenol.* As in the previous experiments on sheep heart (Kulka *et al.* 1961), addition of dinitrophenol to liver homogenates affected the reduction of acetoacetate whenever it affected respiration (Table 10). An increase in respiration was accompanied by a decrease in the yield of β-hydroxybutyrate (Expts. 1 and 2). At lower concentrations of dinitrophenol (10 μM) which did not stimulate respiration appreciably (Expt. 3) there was no major effect on the reduction of acetoacetate. When acetoacetate was the only added substrate 50 μM-dinitrophenol inhibited the O_2 uptake (Expts. 1 and 2).

Action of amytal on the reduction of acetoacetate. Very large effects on the reduction of acetoacetate were caused by amytal (Table 11). When acetoacetate was the only added substrate the rate of reduction was increased more than fourfold in rat liver whereas the respiration was decreased. With acetoacetate and succinate there was also a marked increase in the rate of reduction of acetoacetate. Sheep heart showed similar effects (Table 11).

Amytal and other barbiturates are known to be respiratory inhibitors, affecting the oxidation of most substrates except that of succinate. It blocks the transfer of electrons from $DPNH_2$ to flavoprotein (Jowett & Quastel, 1937; Michaelis & Quastel, 1941; Eiler & McEwen, 1949; Ernster, Jalling, Löw & Lindberg, 1955). If this were the only effect of amytal and if the block were complete [as in certain mitochondrial preparations (Ernster *et al.* 1955)] then it would be expected that in the presence of sufficient amytal the reactions of acetoacetate would be the same as under anaerobic conditions (because O_2, though present, would not be utilizable). In fact the block by amytal is not complete. According to Ernster *et al.* (1955) and Lehninger (1953–54), two pathways of oxidation of $DPNH_2$ exist in animal tissues and only one of these is inhibited by amytal. The relative proportion of the amytal-insensitive pathway in the homogenate used in the present work is shown in Table 12. Irrespective of the substrates used (which did not include succinate) the maximal inhibition was reached when the amytal concentration was between 1 and 6 mM. The residual respiration was 20–30 % of the respiration without amytal and the removal of fumarate, α-oxoglutarate, pyruvate and glutamate was reduced to about the same extent. With citrate the residual substrate removal was still 55 % of that found in the absence of amytal. These experiments show that under the test conditions the amytal-insensitive pathway carried electrons to O_2 at about one-quarter of the rate of electrons transported in the absence of amytal.

In order to examine in detail the metabolic changes occurring in the presence of amytal the intermediate metabolites were measured in an experiment which was set up parallel with that recorded in Table 7 (see Table 13). The main effect of amytal occurred in the absence of suc-

Table 12. *Effect of amytal on the oxygen uptake and substrate removal in rat-liver homogenate*

Incubated at 30° for 60 min.; gas space, O_2. Substrate added: 40 μmoles. Data refer to 4 ml. of 10 % homogenate.

Substrate added	Amytal (mM)	Uptake of O_2 (μmoles)	Substrate removed (μmoles)
None	0	−32·4	—
	0·25	−28·4	—
	1·25	−10·4	—
	6·25	−9·3	—
Fumarate	0	−61·7	−9·5
	0·25	−59·9	−8·9
	1·25	−23·2	−3·2
	6·25	−16·2	−2 8
α-Oxoglutarate	0	−54·8	−25·3
	0·25	−51·9	−30·4
	1·25	−14·5	−5·0
	6·25	−12·1	−5·0
Pyruvate	0	−62·6	−34·0
	0·25	−49·2	−27·0
	1·25	−14·0	−19·8
	6·25	−12·8	−7·6
Glutamate	0	−53·7	−14·7
	0·25	−51·2	−18·7
	1·25	−17·3	−5·3
	6·25	−15·2	−3·2
Citrate	0	−53·5	−22·4
	0·25	−46·5	−26·4
	1·25	−13·7	−12·8
	6·25	−13·4	−12·3

Table 13. *Effect of amytal on oxidations in rat liver*

The experiment was carried out on the same liver and under the same conditions as those of Table 7. Amytal, 2·5 mM.

Substrates added ...	Metabolic changes (μmoles)			
	None	Acetoacetate (40 μmoles)	Acetoacetate (40 μmoles) and succinate (160 μmoles)	Succinate (160 μmoles)
O_2	−10·5	−10·6	− 74·8	− 78·5
Acetoacetate	0	− 8·6	− 17·1	0
β-Hydroxybutyrate	0	+ 9·8	+ 16·0	+ 0·5
Succinate	0	0	−137·2	−133·8
Fumarate + malate	0	0	+100·9	+ 97·8
α-Oxoglutarate	+ 0·15	+ 0·33	+ 0·49	+ 0·73
Citrate	0	0	+ 4·54	+ 0·42
Pyruvate	0	0	+ 3·02	+ 1·04

Table 14. *Balance sheet of the reactions taking place in rat-liver homogenate anaerobically on addition of citrate and acetoacetate*

Data refer to 4 ml. of homogenate containing 10% of tissue. Free CO_2 formed was measured manometrically and bound CO_2 was determined at the end of the incubation period by adding 1 ml. of N-HCl from a side bulb. The initial amounts of bound CO_2 (5·75, 5·04 and 5·08 μmoles respectively in cups 1–3) were determined in separate cups and their values have been deducted in the data given below. Temp., 30°. Gas phase, N_2. Yellow phosphorus in centre well. Incubation time, 60 min.

Vessel no.	Changes		
	1	2	3
Acetoacetate added (μmoles) ...	40·9	0	40·9
Citrate added (μmoles)	0	36·2	36·2
Acetoacetate	−8·90	0	−31·20
β-Hydroxybutyrate	+9·45	+0·63	+33·70
Free CO_2	+3·30	+2·23	+17·6
Bound CO_2	+0·50	−0·19	+14·1
Total CO_2	+3·80	+2·04	+31·7
Citrate	0	−3·4	−15·4
Succinate	+0·39	−0·21	+11·4
α-Oxoglutarate	0	0	+ 3·52
L-Glutamate	−1·11	−0·84	+ 1·54
Fumarate, malate, pyruvate	0	0	0

cinate and consisted of an inhibition of about 75% of the O_2 uptake and an eight- to nine-fold increase in the removal of acetoacetate and the formation of β-hydroxybutyrate. The non-accumulation of other intermediates suggests that the substrate changes of respiration (mainly those of the tricarboxylic acid cycle) proceeded in the normal order though at a reduced rate, with acetoacetate instead of O_2 serving as an additional terminal electron acceptor. When succinate was present, the amounts of intermediates formed were not appreciably changed by amytal (except for β-hydroxybutyrate).

Anaerobic interaction between citrate and acetoacetate

In an earlier paper (Krebs & Eggleston, 1945) it was stated that the tricarboxylic acids did not accelerate reduction of acetoacetate in animal tissues. As shown in Table 9, Expts. 5 and 7, this statement is not valid, citrate being in fact a very rapid hydrogen donor. Thus all dehydrogenases of the tricarboxylic acid cycle involve pyridine nucleotides, i.e. all oxidative steps except that leading from succinate to fumarate can provide hydrogen atoms for the reduction of acetoacetate. It is noteworthy that the rapid reaction of citrate occurs in liver homogenates although most of the *iso*citric dehydrogenase of liver is triphosphopyridine nucleotide-linked. The rapid rate indicates that either the transhydrogenase activity of the tissue is high or that the diphosphopyridine nucleotide (DPN)-linked *iso*citric dehydrogenase is also very active (see Ernster & Glasky, 1960; Ernster, 1960).

A full analysis of the products of the interaction between citrate and acetoacetate (Table 14) shows that the main reaction can be described by the scheme:

2 Acetoacetate + citrate → 2 β-hydroxybutyrate + 2 CO_2 + succinate.

The data are in general agreement with this, the only appreciable deviation being a somewhat low value for succinate and the formation of some α-oxoglutarate and glutamate. The presence of these products indicates that the following reactions also occurred:

$$\text{Acetoacetate} + \text{citrate} \rightarrow \beta\text{-hydroxybutyrate} + \alpha\text{-oxoglutarate} + CO_2$$

$$\text{Citrate} + NH_2 \rightarrow \text{glutamate} + CO_2$$

(Krebs, Eggleston & Hems, 1948; Bartley, Sobrinho-Simões, Notton & Montesi, 1959). In addition some acetoacetate must have been reduced at the expense of endogenous reducing substrates, the presence of which is indicated by the data obtained with vessel 1.

Maximum rates of anaerobic reduction of acetoacetate

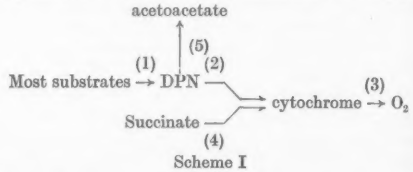
In relation to the role of the reduction of acetoacetate in electron transport (see Discussion) information is required on the maximum rates at which acetoacetate can be reduced anaerobically. From the experiments already reported in Table 9 it follows that the reduction can be coupled with the oxidation of citrate, of fumarate, of pyruvate and of glutamate, Maximum rates may therefore be expected when several substrates are available. This proved to be the case (Table 15). Combinations, e.g. of citrate and fumarate, gave higher β-hydroxybutyrate values than the separate substrates but the effects were not fully additive. The β-hydroxybutyrate yield again exceeded the acetoacetate removal, especially in the presence of pyruvate.

Effect of creatine phosphate

If the reversal of oxidative phosphorylation played a role in the reduction of DPN, and through DPN of acetoacetate, addition of adenosine triphosphate or creatine phosphate would be expected to increase the rate of β-hydroxybutyrate formation. To test this, Expt. 1 (Table 10) was repeated with the addition of creatine phosphate (0·01 M final concentration). This had no effect in the absence of dinitrophenol. In its presence it abolished completely the inhibition of respiration. The reduction of β-hydroxybutyrate was unaffected. These results argue against a role of energy-rich phosphates in the reduction of acetoacetate (see Discussion). The fact that creatine phosphate counteracted the inhibition of respiration by dinitrophenol is in accordance with the view that this inhibition is connected with a fall in concentration of adenosine triphosphate in the presence of dinitrophenol.

DISCUSSION

Aerobic reduction of acetoacetate. All observations on the rate of reduction of acetoacetate described in this paper can be accounted for by the hypothesis put forward previously (Kulka *et al.* 1961). This is based on Scheme I, which illustrates diagrammatically the relevant stages of electron transport between substrates and oxygen, including the joint pathway between cytochrome and oxygen.



Scheme I

If both acetoacetate and β-hydroxybutyric dehydrogenase are present a reduction of acetoacetate occurs when the transfer of electrons to DPN (reaction 1) is more rapid than the transfer of electrons from DPN to oxygen (reactions 2 and 3). Agents which have been found to affect the aerobic yield of β-hydroxybutyrate in a major way are succinate, amytal and dinitrophenol. Succinate accelerates the reduction of acetoacetate because reaction (4) is more rapid than reaction (2), and the electrons from succinate therefore compete successfully with those from $DPNH_2$. They thus inhibit competitively the transfer of electrons from $DPNH_2$ to oxygen. At the same time succinate, because of its rapid oxidation, provides hydrogen donors for DPN such as malate, pyruvate, *iso*-citrate and α-oxoglutarate. As shown in anaerobic experiments (Table 9), these oxidation products of succinate readily reduce acetoacetate.

Amytal stimulates β-hydroxybutyrate formation because it inhibits reaction (2) without blocking it completely. It still permits the oxidative formation of reducing agents through the tricarboxylic acid cycle. Dinitrophenol, on the other hand, decreases the yield of β-hydroxybutyrate because it accelerates reactions (2) and (3) and therefore decreases the amount of $DPNH_2$ available for reaction (5).

As the reduction of β-hydroxybutyrate is preceded by the reduction of DPN the observations by Chance and others (Chance & Williams, 1955; Chance & Hollunger, 1960; Klingenberg, Slenczka & Ritt, 1959; Birt & Bartley, 1960) on the rise of the concentration of $DPNH_2$ on addition of succinate are related to the findings reported in this paper. In fact the formation of β-hydroxybutyrate is a magnified reflexion of the formation of $DPNH_2$.

The explanation of the succinate effect offered here differs from that put forward by Chance & Hollunger (1960), who assume that the increased

rate of reduction of DPN in the presence of succinate involves energy-rich intermediates. They suggest that adenosine triphosphate or possibly enzyme-bound, energy-rich phosphate formed as a result of the oxidation of succinate causes a reversal of oxidative phosphorylation. The coupling of phosphorylation with the oxidation of $DPNH_2$ may be formulated:

$$DPNH_2 + FAD + ADP + P \rightleftharpoons DPN + FADH_2 + ATP \quad (6)$$

(where FAD and $FADH_2$ represent flavoprotein and reduced flavoprotein, ADP is adenosine diphosphate and ATP adenosine triphosphate). If this reaction is reversible any increase in the concentration of adenosine triphosphate would drive the reaction from right to left. There is evidence [the occurrence of the adenosine triphosphate–phosphate-exchange reaction (Boyer, Luchsinger & Falcone, 1956; Cooper & Lehninger, 1957; Löw, Siekevitz, Ernster & Lindberg, 1958)] that reaction (6) is in fact reversible. The mechanism proposed by Chance & Hollunger is therefore a feasible one. However, it cannot be responsible for the reduction of DPN in the present experiments because reaction (6) is completely inhibited by higher concentrations of amytal (Löw *et al.* 1958). The fact that amytal, on the contrary, greatly stimulates acetoacetate reduction is incompatible with the hypothesis of Chance & Hollunger, and supports the concept favoured in the present paper. The failure of creatine phosphate to promote the reduction of acetoacetate also argues against the involvement of energy-rich phosphate.

Anaerobic reduction of acetoacetate. Anaerobically only reactions (1) and (5) are expected to occur. The data on the anaerobic rate of formation of β-hydroxybutyrate show that (1) and (5) are relatively slow when no substrate has been added to the liver homogenate. Fumarate (malate), citrate (*iso*citrate), α-oxoglutarate, glutamate and pyruvate greatly accelerate the reduction of acetoacetate whereas lactate has a slight effect. The low rates of reduction in the unsupplemented homogenate and the fact that most of the mitochondrial pyridine nucleotide is normally present in the oxidized form must be ascribed to the low concentrations of substrates capable of reducing DPN, i.e. of intermediates of the tricarboxylic acid cycle and of related reactions. The only intermediate which may occur in relatively high concentrations is lactate and this is not an effective reducing agent even at high concentrations. This is presumably due to the location of lactic dehydrogenase in relation to DPN within the cell's compartments which does not permit the effective coupling with the mitochondrial DPN or the carriers between the DPN of mitochondria and other regions.

Effect of oxygen. Oxygen increased the rate of reduction of acetoacetate in the presence of succinate but decreased it in the presence of fumarate and citrate. This decrease can also be explained on the basis of Scheme I. The rate of reduction of acetoacetate depends on the amounts of available $DPNH_2$, and these in turn depend on the rate of generation of $DPNH_2$ (reaction 1) and the rate of reaction (2). Given an excess of reducing substrate such as fumarate or citrate, reaction (1) may be expected to be the same aerobically and anaerobically but reaction (2) is zero anaerobically and greater than zero aerobically. Hence aerobically less $DPNH_2$ will be available for reaction (5). The inhibition by oxygen of the reduction of acetoacetate can thus be ascribed to a competition of the electron-transport chain with acetoacetate for $DPNH_2$. This situation applies to all pyridine nucleotide-linked substrates. In the presence of glutamate and α-oxoglutarate, which yield succinate, the position is less clear-cut because of the inhibition of reaction (2) by the succinate formed. It is therefore not unexpected that oxygen does not appreciably decrease the reduction of acetoacetate in the presence of glutamate or α-oxoglutarate.

Partial inhibition of electron transport by amytal. According to Jalling, Lindberg & Ernster (1955), 1·8 mM-amytal inhibits the mitochondrial oxidation of pyruvate, citrate and malate completely. In the homogenates used in the present experiments (and in minced material not homogenized; unpublished experiments) the inhibition of respiration in the presence of these substrates was no more than 75%, indicating that about 25% of tissue respiration passed through the 'extramitochondrial' pathway. The fact that the inhibition of substrate utilization by amytal was lowest with citrate (45%) is in agreement with the well-established observation that a large proportion of *iso*citric dehydrogenase resides extramitochondrially. The partial inhibition of tissue respiration suggests that at least two enzymes catalysing the same step but differing in the sensitivity to amytal exist in the tissue preparations. The enzymes may either differ in their location and accessibility or in their physicochemical properties. Multiple enzymes ('isoenzymes'; Markert & Møller, 1959) have in fact been discovered in liver and other tissues (Delbrück, Schimassek, Bartsch & Bücher, 1959; Wieland, Pfleiderer, Haupt & Worner, 1959; Thorne, 1960; Wieme, 1959).

Physiological significance of the reduction of acetoacetate. The question arises of the physiological significance of the ready reduction of acetoacetate. Devlin & Bedell (1960) found that $DPNH_2$ added to mitochondria is not oxidized but

Table 15. *Maximum anaerobic rates of removal of acetoacetate and formation of β-hydroxybutyrate in rat-liver homogenate*

Rat-liver homogenate, 10%. Temp., 30°. Gas phase, N_2. Incubation time, 20 min. Data refer to 4 ml.

Substrates added (all 40 μmoles)	Acetoacetate removed (μmoles)	β-Hydroxybutyrate formed (μmoles)
Acetoacetate	3·0	5·8
Acetoacetate; fumarate	7·8	8·4
Acetoacetate; pyruvate	6·1	11·9
Acetoacetate; citrate	11·2	13·8
Acetoacetate; citrate; fumarate	16·3	17·9
Acetoacetate; citrate; pyruvate	12·2	17·3
Acetoacetate; citrate; pyruvate; fumarate	13·4	18·6
Acetoacetate; pyruvate; fumarate	14·3	17·9
Pyruvate	0	1·7

reacts with oxygen when catalytic quantities of acetoacetate or β-hydroxybutyrate are present. β-Hydroxybutyrate is taken to be a hydrogen carrier between the 'extramitochondrial' and 'intramitochondrial' regions. If acetoacetate played a major role as a hydrogen carrier the rate of its reduction, and of the reoxidation of β-hydroxybutyrate, should be of the same order of magnitude as the overall rates of respiration.

The maximum figures for the reduction of acetoacetate in Table 15 (16 μmoles) represent a rate of 120 μmoles/g. wet wt./hr. at 30°. The corresponding rate of consumption of oxygen under similar conditions was about 115 μmoles (calculated from data in Table 9). As 1 oxygen molecule is equivalent to 2 acetoacetate molecules the observed maximum rate of reduction would be sufficient for about half the electron transport to pass through the acetoacetate–β-hydroxybutyrate system. Since only the pyridine nucleotide-linked dehydrogenases, not succinic dehydrogenase, would be coupled to the acetoacetate system, five of six of the electrons reaching oxygen could maximally involve acetoacetate when carbohydrate serves as the substrate. These considerations lead to the conclusion that a substantial part but not the whole of the electron transport in liver homogenate could pass through acetoacetate. In liver the rates would be adequate for dealing with any $DPNH_2$ generated in the cytoplasm, e.g. by *iso*citric or triose phosphate dehydrogenases. It should be mentioned that analogous roles as hydrogen carriers have been considered for the glycerophosphate–dihydroxyacetone systems (Estabrook & Sacktor, 1958; Lee, Takemori & Lardy, 1959; Bücher & Klingenberg, 1958; Zebe, Delbrück & Bücher, 1959) and for the malate–oxaloacetate system (Delbrück *et al.* 1959; Szent-Gyorgyi, 1935).

SUMMARY

1. In many animal tissues (see Table 2) succinate accelerated the aerobic rate of reduction of acetoacetate as previously demonstrated for sheep heart. This effect was especially pronounced in liver.

2. Acetoacetate served as a major substrate of respiration in the diaphragm of various species and also in epididymal fat, testis and brain of the rat.

3. The determination of intermediate metabolites formed in respiring sheep heart and rat liver on addition of succinate and acetoacetate indicates that succinate was oxidized in preference to all other substrates.

4. Under conditions where 2:4-dinitrophenol stimulated respiration the rate of reduction of acetoacetate was diminished.

5. Amytal greatly increased the aerobic rate of reduction of acetoacetate in rat liver and sheep heart.

6. In liver homogenates the inhibition of respiration by amytal did not exceed 75%. The remaining 25% represent the capacity of the amytal-insensitive or 'extramitochondrial' pathway of electron transport.

7. Anaerobically fumarate, glutamate, α-oxoglutarate, pyruvate and citrate accelerated the rate of reduction of acetoacetate in rat-liver homogenates. With these substrates the aerobic rates of reduction were lower than the anaerobic rates. Succinate was the only substrate in rat liver that increased the rate aerobically but not anaerobically.

8. Citrate reacted anaerobically with acetoacetate according to the scheme:

2 Acetoacetate + citrate → 2 β-hydroxybutyrate + 2 carbon dioxide + succinate.

9. Maximum rates for the reduction of acetoacetate were found on addition of mixtures of substrates such as citrate plus fumarate. The rates measured permit the assumption (Devlin & Bedell, 1960) that in the respiration of rat liver a substantial part of the electrons reaching oxygen passes through the acetoacetate–β-hydroxybutyrate system.

10. Creatine phosphate had no effect on the rates of acetoacetate reduction but abolished the inhibition of respiration by 2:4-dinitrophenol.

11. The observations on the formation of β-hydroxybutyrate from acetoacetate summarized in paragraphs 1, 4, 5, 7 and 10 are in agreement with Scheme I, according to which the reduction takes place whenever reaction (1) is more rapid than reaction (2).

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Oligosaccharides in Dextran-Producing Cultures of *Streptococcus bovis*

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Species of *Leuconostoc mesenteroides* and *Streptococcus bovis* produce dextran and fructose when grown in media containing sucrose (Hehre, 1951; Tsuchiya *et al.* 1952; Bailey, Barker, Bourne & Stacey, 1957; Dain, Neal & Seely, 1956; Bailey & Oxford, 1958*a*). In addition, reducing disaccharides containing glucose and fructose units are formed. One of these, leucrose (5-*O*-D-glucopyranosyl-D-fructopyranose), has been isolated from cultures of *L. mesenteroides* (NRRL B-512F) and characterized by Stodola, Sharpe & Koepsell (1956), who also mentioned the formation of isomaltulose (Sharpe, Stodola & Koepsell, 1954). Bailey & Bourne (1959) have isolated leucrose from dextran-producing cultures of *S. bovis* and noted that a second disaccharide was produced. The present paper reports a closer examination of the oligosaccharides produced in cultures of *S. bovis*.

EXPERIMENTAL

Organism. *Streptococcus bovis* (strain I; Bailey & Oxford, 1958*a*, *b*) was used.

Culture medium. The organism was grown in a medium containing yeast extract (1%, w/v), Bacto-tryptose (1·5%, w/v), sucrose (8·5%, w/v) and separately sterilized potassium carbonate (0·004M), pantothenic acid (20 mg./l.) and biotin (0·01 mg./l.). A 1 l. culture was incubated at 37° for 24 hr., when more sucrose (30 g.) and sodium bicarbonate (1 g.) were added. The pH of the medium was adjusted to 6·5 with N-NaOH and the culture incubated for a further 3 days.

Paper chromatography. Whatman no. 1 filter paper was used. The solvent systems were: (*a*) organic phase of ethyl acetate–water–pyridine (2:2:1, by vol.); (*b*) ethyl acetate–acetic acid–water (9:2:2, by vol.); (*c*) butanol–benzene–pyridine–water (5:1:3:2, by vol.); (*d*) water-saturated butanol.

Paper ionophoresis. Ionophoresis was conducted on Whatman no. 3 MM filter paper in 0·1M-borate, pH 10 (Foster, 1953), and 0·1M-molybdate, pH 5·5 (Bourne, Hutson & Weigel, 1959), at 80 v/cm. for about 1 hr.

Selective spray reagents. The spray reagents used were: (*a*) acetone–silver nitrate–ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950), for the general detection of carbohydrates; (*b*) urea–phosphoric acid (Wise, Dimler, Davis & Rist, 1955), for the detection of ketoses, particularly compounds containing a leucrose unit, which stain a dull brown, strongly fluorescent in u.v. light; (*c*) aniline–diphenylamine–phosphoric acid (Schwimmer & Bevenue, 1956), found to give a specific green spot with 6-*O*-substituted glucose (e.g. isomaltose); (*d*) triphenyltetrazolium chloride (Feingold, Avigad & Hestrin, 1956), reaction with which was regarded as evidence that reducing glucose units were unsubstituted at the C-2 hydroxyl group.

Reduction of aldoses and ketoses. Aqueous solutions of the sugars (about 1%) were mixed with an equal volume of potassium borohydride solution (1%) and allowed to stand overnight (Bragg & Hough, 1957). The solutions were deionized by treatment with Amberlite resin IR-120 (H^+) followed by evaporation to dryness and repeated distillation with dry methanol. Completion of reduction was checked by ionophoresis in molybdate solution (Bourne *et al.* 1959). The product was always purified by paper chromatography.

Acid hydrolysis. Hydrolysis was effected at 96° in 0·5N-sulphuric acid for 3 hr. (complete) or 1 hr. (partial). The solutions were deionized by treatment with Amberlite resin IRA-400 (carbonate).

RESULTS

Isolation of oligosaccharides

At the end of the incubation period dextran was removed from the culture fluid (1 l.) by precipitation with acetone (2 vol.). The supernatant liquid was evaporated to 200 ml. and fractionated on a charcoal–Celite column (40 cm. × 7 cm.; Whistler & Durso, 1950). Water eluted fructose and a small amount of glucose. Di- and tri-saccharides were eluted with aqueous 1–6% and 6–10% ethanol respectively. A small quantity of material eluted with aqueous 15% ethanol was not examined further.

Leucrose (m.p. 156°, mixed m.p. 156°) was crystallized from the disaccharide fraction in ethyl acetate (Bailey & Bourne, 1959). The mother liquor was fractionated on a cellulose column (Whatman cellulose powder, standard grade; 60 cm. × 4·5 cm.) with solvent (*c*) as the irrigant. The fraction containing disaccharide A was concentrated, dissolved in water and freeze-dried. From the fraction containing leucrose a disaccharide B was isolated in trace quantity by paper chromatography with solvent (*b*).

Paper chromatography of the trisaccharide fraction (300 mg.) with solvent (*a*) revealed a trace quantity of a trisaccharide A, which was isolated by this method. The main trisaccharide fraction then appeared to be homogeneous in the four chromatography solvents, but 100 mg. was resolved into two components, trisaccharide B (about 50 mg.) and C (about 40 mg.), by paper ionophoresis in borate solution. The yields of oligosaccharides from a 1 l. culture are shown in Table 1.

Structural examination of the oligosaccharides

Disaccharide A. Disaccharide A stained blue with spray (*b*) (ketose-type) and had the characteristics shown in Table 2. Its high rate of mobility during ionophoresis in molybdate solution was indicative of a 6-*O*-substituted fructose (Bourne, Hutson & Weigel, 1960). Acid hydrolysis gave glucose and fructose (paper chromatography), shown by elution and determination with anthrone (Yemm & Willis, 1954) to be present in the ratio 1 : 0·75. Determination of glucose and fructose in an artificial mixture (ratio 1 : 1), treated with acid in the same way as the disaccharide, revealed a glucose/fructose ratio of 1 : 0·81.

Disaccharide A was reduced with potassium borohydride (Bragg & Hough, 1957). Ionophoresis in molybdate solution (pH 5·5) showed that the alcohol migrated as a single component, with

Table 1. *Yields of oligosaccharides from a* 1 *l. culture of* Streptococcus bovis *grown on sucrose* (115 *g.*)

The expected yield of dextran would be about 40 g.

Compound	Yield
Leucrose	1·8 g.
Disaccharide A (isomaltulose)	250 mg.
Disaccharide B (isomaltose)	Trace
Trisaccharide A (isomaltotriulose)	Trace
Trisaccharide B (isomaltotriose)	150 mg.
Trisaccharide C (5-*O*-α-isomaltosyl-D-fructose)	120 mg.

mobility referred to sorbitol (M_s) 0·8 (identical with that of a 5- or 6-*O*-glycosyl-sorbitol or -mannitol), and that on hydrolysis it gave glucose and hexitol but not fructose.

When estimated by the Shaffer & Hartmann (1921) method disaccharide A (4 mg.) had a reducing power equivalent to 1·84 mg. of fructose. This corresponded to 87·7 % of the calculated value for a disaccharide.

Disaccharide A was incubated with almond β-glycosidase at 25° for 24 hr. Paper chromatography showed that no hydrolysis had occurred. Under the same conditions 1-*O*-β-D-glucopyranosyl-D-fructose was completely hydrolysed.

Disaccharide A (50 mg.) was converted into its phenylosazone (30 mg.) by the method of Avigad (1959). The recrystallized phenylosazone had m.p. 171–173°. Admixture with isomaltose phenylosazone, m.p. 173–175°, caused no depression in melting point. The phenylosazone behaved as a pure disaccharide phenylosazone during chromatography in solvents (*a*) and (*d*). Its infrared spectrum was identical with that of isomaltose phenylosazone. Thus disaccharide A was isomaltulose.

Disaccharide B. Disaccharide B could be detected on paper chromatograms and ionophoretograms with spray reagents (*a*), (*c*) and (*d*), and was indistinguishable from isomaltose (Table 2). Spray (*c*) gave a green spot characteristic for a 6-*O*-substituted glucose. Acid hydrolysis gave only glucose (paper chromatography). Thus the trace disaccharide B was almost certainly isomaltose.

Trisaccharide A. Trisaccharide A could be detected on paper chromatograms and ionophoretograms (Table 2) with the spray reagents (*a*) and (*b*). Chromatography and ionophoresis of the complete acid hydrolysate revealed the presence of components identical with glucose and fructose (small amount), and a partial hydrolysate contained also components identical with isomaltose and isomaltulose.

Trisaccharide A alcohol, obtained by reduction of trisaccharide A with potassium borohydride (Bragg & Hough, 1957), had M_s 0·75, identical with that of 6-*O*-isomaltosylsorbitol and different from all but 5- or 6-*O*-biosyl-sorbitols or -mannitols. Complete hydrolysis of the alcohol gave components identical with glucose and a hexitol; a partial hydrolysate contained isomaltose and glucose as the only reducing components. Thus trisaccharide A was most probably isomaltotriulose.

Trisaccharide B. Trisaccharide B could be detected on paper chromatograms and ionophoretograms with spray reagents (*a*), (*c*) (characteristic green for a 6-*O*-substituted glucose) and (*d*). It was indistinguishable from isomaltotriose (Table 2). Complete acid hydrolysis gave only glucose, whereas a partial acid hydrolysis gave isomaltose and glucose. It was not hydrolysed by almond β-glycosidase.

Trisaccharide B was reduced with potassium borohydride (Bragg & Hough, 1957). Ionophoresis in molybdate solution (pH 5·5) showed that the alcohol migrated at the same rate as 6-*O*-isomaltosylsorbitol (M_s 0·75; see above). Complete hydrolysis of the alcohol gave glucose and a hexitol. A partial hydrolysate of the alcohol was shown, by chromatography in solvents (*a*) and (*b*) and ionophoresis in molybdate solution (pH 5·5), to contain two reducing components identical with glucose and isomaltose, and two non-reducing components identical with a hexitol and 6-*O*-glucopyranosylsorbitol, as well as the unchanged alcohol.

Table 2. *Properties of some oligosaccharides compared with those isolated from* Streptococcus bovis *cultures*

Solvents: (*a*) organic phase of ethyl acetate–water–pyridine (2:2:1, by vol.); (*b*) ethyl acetate–acetic acid–water (9:2:2, by vol.); (*c*) butanol–benzene–pyridine–water (5:1:3:2, by vol.); (*d*) water-saturated butanol.

	Chromatography R_G				Ionophoresis		
Compound	Solvent (*a*)	Solvent (*b*)	Solvent (*c*)	Solvent (*d*)	M_G (borate)	M_S (molybdate)	Phenylosazone (m.p.)
1-*O*-β-Glucopyranosyl-fructose	0·59	0·41	0·57	0·28	0·74	0·25	—
Sucrose	0·74	0·63	0·81	0·50	0·10	0	—
Turanose	0·75	0·54	0·81	0·51	0·69	0·1	202–204°
Maltulose	0·63	0·44	0·64	0·39	0·63	0·15	202–204
Leucrose	0·55	0·50	0·50	0·32	0·56	0·35	186–188
Isomaltulose	0·64	0·50	0·68	0·33	0·60	0·64	173–175
Isomaltose	0·58	0·27	0·50	0·31	0·70	0	173–175
Isomaltotriose	0·35	0·15	—	0·10	0·58	0	—
Disaccharide A	0·63	0·50	0·69	0·35	0·61	0·65	171–173
Disaccharide B	0·59	0·28	0·50	0·31	0·70	0	—
Trisaccharide A	0·41	0·24	—	0·15	0·50	0·47	—
Trisaccharide B	0·35	0·15	—	0·10	0·57	0	—
Trisaccharide C	0·35	0·15	—	0·10	0·44	0·30	—

Trisaccharide C. Trisaccharide C could be detected on paper chromatograms and ionograms (Table 2) with spray reagent (*b*) as a brown spot which fluoresced in u.v. light (as did leucrose). Acid hydrolysis yielded glucose and a smaller quantity of fructose (paper chromatography). A partial hydrolysate contained glucose, isomaltose and small amounts of leucrose and fructose, as well as the unchanged trisaccharide. Chromatography of an equimolar mixture of isomaltose and leucrose, after the same treatment with acid, gave a similar pattern.

Trisaccharide C was reduced with potassium borohydride (Bragg & Hough, 1957). During ionophoresis in molybdate solution (pH 5·5) the product migrated with M_g 0·75 (see above). Complete acid hydrolysis of the alcohol gave glucose as the only reducing hexose together with a hexitol. Chromatography and ionophoresis (molybdate) of a partial hydrolysate of the alcohol revealed the presence of components identical with glucose, a hexitol, isomaltose, 5- or 6-*O*-glycosylsorbitol and unchanged material.

Trisaccharide C was not hydrolysed by almond β-glycosidase; thus it was most probably O-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 5)-D-fructose (5-*O*-α-isomaltosyl-D-fructose).

Action of partially purified Streptococcus bovis *dextransucrase on leucrose in the presence of sucrose*

Leucrose (50 mg.) and sucrose (50 mg.) were incubated at 37° with dextransucrase (2 mg.) in 0·05 M-acetate buffer (pH 5·2, 2 ml.) for 24 hr. The dextransucrase preparation was supplied by Dr R. W. Bailey and prepared according to Bailey (1959). Chromatography of the digest revealed a small amount of a component with R_G 0·35, 0·15 and 0·10 in solvents (*a*), (*b*) and (*d*) respectively. It could be detected with spray reagents (*a*) and (*b*). The latter gave a brown spot which fluoresced strongly in u.v. light. Some of this component was isolated by paper chromatography and subjected to the same analysis as trisaccharide C. In all these tests the compound was identical with trisaccharide C. The enzyme did not produce detectable amounts of this trisaccharide from leucrose or sucrose separately. This confirmed the structure assigned to the trisaccharide.

DISCUSSION

The evidence presented here and by Bailey & Bourne (1959) characterizes three of the oligosaccharides in *S. bovis* cultures as isomaltulose, leucrose and *O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 5)-D-fructose (5-*O*-α-isomaltosyl-D-fructose); other products were almost certainly isomaltose, isomaltotriose and isomaltotriulose. A large quantity of fructose and a smaller amount of glucose were also present; all of the sucrose had been utilized.

It has been shown that certain simple sugars can act as receptors of transferred glucosyl units when dextransucrase preparations from *L. mesenteroides* (NRRL B-512) (Koepsell *et al.* 1953), *L. mesenteroides* (Birmingham) (Bailey *et al.* 1957) and *S. bovis* (Bailey, 1959) act on sucrose. Such sugars lead to the synthesis of oligosaccharides. Isomaltose and isomaltotriose were found, in the *Leuconostoc* system, to be good receptors whereas glucose, fructose and leucrose acted as poor receptors. The present work shows that the same is true for the *S. bovis* dextransucrase system.

Glucosyl transfer of the above type can occur during dextran synthesis and be responsible for by-products. The amount of such by-products will depend not only on the efficiency of the receptor molecules but also on their relative concentrations. Thus transfer to fructose, which is a poor receptor but present in large quantities, results in relatively large amounts of leucrose (mainly) and isomaltulose. Transfer to glucose would result in much less isomaltose. The concentrations of disaccharides could increase until they competed with fructose as receptors, thus leading to 5-*O*-α-isomaltosyl-D-fructose and isomaltotriulose. The production of isomaltotriose is almost certainly via isomaltose. That the latter was found only in very small amount is reasonable as it is a good receptor and would be soon converted into isomaltotriose. It is interesting that fructose appears to act as a receptor in both the pyranose and furanose forms, to give leucrose and isomaltulose respectively, the former being preferred.

After the present work had been completed, Sharpe, Stodola & Koepsell (1960) confirmed the formation of isomaltulose as a by-product during the synthesis of dextran by enzyme preparations of *L. mesenteroides* (NRRL B-512F).

SUMMARY

1. *Streptococcus bovis*, when grown in a medium containing sucrose, has been shown to produce leucrose, isomaltulose, isomaltose, isomaltotriose, 5-*O*-α-isomaltosyl-D-fructose and isomaltotriulose.

2. The mechanism of the formation of these oligosaccharides is discussed.

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The Effect of Hind-Limb Ischaemia on the Products and Intermediates of Glycolysis in Rat Tissues

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In small mammals the survival period after fatal limb ischaemia can be divided into two parts. In the first phase, under ordinary environmental conditions (air temperature 18–22°), the temperatures of the main heat-producing organs in the rat, the liver and brain, fall before their oxygenation is impaired (Stoner, 1958*a*). This has been interpreted as an effect of injury on the control of heat production (see Stoner & Threlfall, 1960; Stoner, 1960). In the second phase, when the animal is dying, the physiological and biochemical changes are dominated by the gradually increasing hypoxia of the tissues. Our interest has been focused on the first stage and attempts to elucidate the mechanism of its changes have so far yielded the following information.

The injured rat's condition cannot be explained by depletion of the stores of energy-rich phosphates (Goranson, Hamilton & Haist, 1948; Stoner & Threlfall, 1954; Threlfall & Stoner, 1957). After injury the catabolism of glucose is inhibited in the early stages of its breakdown and in the oxidation of the pyruvate formed from it (Stoner, Heath & Collins, 1960). At the same time it would appear that ischaemic limb injury does not damage liver mitochondria, in so far as this can be judged from their behaviour *in vitro* after isolation (Aldridge & Stoner, 1960).

We have now investigated the concentrations of glycolytic products and intermediates in the tissues during the first part of the survival period, determining them chromatographically (except for lactate) since the methods used previously (LePage, 1946; Threlfall & Stoner, 1954) were inadequate. The effect of hind-limb ischaemia has been studied under different environmental and endocrinological conditions to determine the effect of varying the intensity of the response to injury. Some of the results have been mentioned in reviews (Stoner & Threlfall, 1960; Stoner, 1960).

METHODS

Animal techniques. Male albino rats (242±36 g. body wt.) of the Porton strain were used. M.R.C. diet 41 B (Bruce & Parkes, 1956) was available until the start of the experiment except when 'fasting' rats were used, when it was removed from the cages on the day before the experiment.

The methods for producing limb ischaemia, cold-acclimatization, adrenalectomy and adrenal-medullectomy and altering environmental conditions have been described previously (Stoner, 1958*a*, *b*). The cold-acclimatized rats were given supplementary ascorbic acid (150 mg. by mouth two or three times/week) and used after they had been at 3° for 58–77 days. Adrenalectomized rats, given 1% (w/v) NaCl to drink, were used 3–4 days after operation. Adrenal-medullectomized rats were used 50–103 days after operation. Liver samples were obtained from guillotined rats or rats anaesthetized with sodium pentobarbitone [5 mg. of Veterinary Nembutal (Abbott)/100 g. body wt., intraperitoneally]. Muscle samples were taken under sodium pentobarbitone anaesthesia. In all cases the samples were frozen immediately in liquid N_2.

Analytical methods. The phosphorylated glycolytic intermediates were estimated as previously described (Threlfall, 1957), the equivalent of 0·5 g. of each tissue being used for chromatographic separation. Glucose 6-phosphate and fructose 6-phosphate were not adequately separated by the solvent systems used and were pooled. This mixture is described in the text as hexose 6-phosphate. Lactate was determined by the method of Barker & Summerson (1941). Pyruvate and α-oxoglutarate were determined chromatographically with the solvent system of El Hawary & Thompson (1953) on extracts prepared in a Nelco 10 homogenizer by the method of Cavallini & Frontali (1954). The control values were similar to those obtained by these workers and by Neish (1953).

Results have been expressed as μmoles of compound/g. wet wt. of tissue and where possible are shown as mean ± standard deviation. The changes found in muscle could not be accounted for by alterations in its water content (Threlfall & Stoner, 1957). The size and water content of the liver were unaffected by limb ischaemia. In 12 intact control rats the liver weight was 3·4 ± 0·69/100 g. body wt. and its mean water content 71·2 ± 0·5%. In 12 intact rats 3 hr. after a 4 hr. period of bilateral hind-limb ischaemia the corresponding values were 3·2 ± 0·43/100 g. body wt. and 71·2 ± 0·8%. When possible statistical comparison of the means has been carried out according to Student's *t* test as modified by Fisher (1934) for small samples.

RESULTS

The physiological disturbance in the rat after bilateral hind-limb ischaemia for 4 hr. has been described previously (Threlfall & Stoner, 1954, 1957; Stoner, 1958*a*, *b*; Stoner & Threlfall, 1960). In fed rats at an air temperature of 18–22° this injury has an 85% mortality rate with a survival

Table 1. *Phosphorylated glycolytic intermediates in injured muscle*

Effect of a 4 hr. period of bilateral hind-limb ischaemia in fed rats on the phosphorylated glycolytic intermediates in the injured hind-limb muscle and uninjured fore-limb muscle. Samples were obtained under sodium pentobarbitone anaesthesia. Number of rats is shown in parentheses. Environmental temperature 18–22°.

Compound	Controls	End of hind-limb ischaemia	3 hr. after hind-limb ischaemia
		(μmoles/g. wet wt. mean ± S.D.)	
		Injured muscle	
Glucose 1-phosphate	0·20 ± 0·11 (7)	0·41 ± 0·48 (5)	0·18 ± 0·11 (6)
Hexose 6-phosphate	1·12 ± 0·37 (8)	2·05 ± 0·81* (5)	0·60 ± 0·21† (6)
Fructose 1:6-diphosphate	0·22 ± 0·06 (8)	0·08 ± 0·03* (5)	0·13 ± 0·08 (6)
Phosphoglycerate	0·13 ± 0·04 (6)	0·19 (2)	0·17 ± 0·07 (5)
α-Glycerophosphate	0·51 ± 0·18 (6)	1·59 ± 0·39* (5)	0·51 ± 0·21 (5)
		Uninjured muscle	
Glucose 1-phosphate	0·15 ± 0·04 (7)	0·23 ± 0·22 (4)	0·25 ± 0·13 (7)
Hexose 6-phosphate	0·99 ± 0·46 (8)	1·72 ± 0·89 (4)	1·46 ± 0·43 (7)
Fructose 1:6-diphosphate	0·25 ± 0·07 (8)	0·27 ± 0·13 (4)	0·35 ± 0·08† (7)
Phosphoglycerate	0·16 ± 0·03 (5)	0·23 (2)	0·18 ± 0·11 (6)
α-Glycerophosphate	0·53 ± 0·14 (5)	0·75 ± 0·40 (3)	0·62 ± 0·22 (5)

* Difference significant at $P < 0·001$.

† Difference significant at $P < 0·05$.

time of about 13 hr. Previous fasting, adrenal-medullectomy, adrenalectomy and raising the environmental temperature all increase the mortality rate to 100% and shorten the survival time, whereas after cold-acclimatization the mortality rate, at an air temperature of 18–22°, is reduced to 23% and the time to death is greatly prolonged in those which die.

Muscle

In the controls there was no difference between the results on fore- and hind-limb samples (Table 1) or on samples taken at different times of the day. The hexose monophosphate concentrations in the control samples were of the same order as those reported by Cori & Cori (1931) and Frunder, Richter, König & Börnig (1959), but lower than those given by Conway & Hingerty (1946), LePage (1948) and Derache, Trémolières, Griffaton & Lowy (1956).

At the end of bilateral hind-limb ischaemia for 4 hr. there were significant increases in the hexose 6-phosphate and α-glycerophosphate concentrations in the injured hind-limb muscle and a fall in its fructose 1:6-diphosphate concentration. There were no significant changes in the fore-limb muscle at this time (Table 1). Three hours after the tourniquets had been removed the only significant changes were a fall in the hexose 6-phosphate concentration in the injured muscle and an increase in that of fructose 1:6-diphosphate in the uninjured muscle (Table 1). The increases in the concentrations of glucose 1-phosphate and glucose 6-phosphate did not quite reach significance at P 0·05. In four medullectomized rats the concentrations of glucose 1-phosphate, hexose 6-phosphate and fructose 1:6-diphosphate in the uninjured muscle 3 hr. after removal of the tourniquets were 0·14 ± 0·08, 0·99 ± 0·49 and 0·22 ± 0·11 μmole/g. wet wt. respectively (medullectomized control values were within the range for intact rats).

The concentrations of the products of glycolysis, together with α-oxoglutarate, in the uninjured fore-limb muscle of intact and medullectomized rats, are shown in Table 2. The pyruvate concentration was higher and the lactate/pyruvate ratio lower than

Table 2. *Concentration of lactate, pyruvate and α-oxoglutarate in rat muscle*

The muscle was removed from the fore-limbs of control intact and medullectomized rats, and from intact rats 3 hr. and medullectomized rats 2·5 hr. after a 4 hr. period of bilateral hind-limb ischaemia. Samples were removed under sodium pentobarbitone anaesthesia. Environmental temperature 19°.

Treatment	Pyruvate [μmoles/g. wet wt. (mean ± S.D.)]	Lactate [μmoles/g. wet wt. (mean ± S.D.)]	α-Oxoglutarate [μmoles/g. wet wt. (mean ± S.D.)]	Lactate/pyruvate ratio (mean ± S.D.)
Control	0·113 ± 0·034 (5)	4·71 ± 1·47 (6)	0·007 ± 0·004 (5)	47 ± 14 (5)
Experimental	0·244 ± 0·088* (6)	6·42 ± 2·81 (6)	0·007 ± 0·002 (6)	27 ± 6* (6)
Control†	0·083 (2)	2·83 (2)	0·010 (2)	34·4 (2)
Experimental†	0·059 ± 0·014 (4)	2·39 ± 0·55 (4)	0·007 ± 0·001 (4)	41 ± 10 (4)

* Significantly different from corresponding control at $P < 0·05$. † Medullectomized.

Table 3. *Phosphorylated glycolytic intermediates in rat liver*

Liver samples were taken from intact rats under sodium pentobarbitone anaesthesia; rats were given access to food up to the start of the experiment. All rats except those killed at 10.0 a.m. were given an ether anaesthetic lasting 3 min. at 10.0 a.m. on the day of sampling. Environmental temperature 18–22°.

Time	Duration of fast (hr.)	No. of rats	Glucose 1-phosphate [μmole/g. wet wt. (mean ± S.D.)]	Hexose 6-phosphate [μmole/g. wet wt. (mean ± S.D.)]	Fructose 1:6-diphosphate [μmole/g. wet wt. (mean ± S.D.)]
10.0 a.m.	0	6	0·38 ± 0·13	0·55 ± 0·17	0·23 ± 0·10
2.0 p.m.	4	6	0·47 ± 0·19	0·83 ± 0·25*	0·29 ± 0·06
5.0 p.m.	7	5	0·53 ± 0·15	0·60 ± 0·12	0·27 ± 0·05
3.0 a.m.	17	2	Not detected	0·33	0·33
10.0 a.m.	24	5	0·12 ± 0·01†	0·68 ± 0·31	0·38 ± 0·09*
2.0 p.m.	28	4	0·12 ± 0·12*	0·46 ± 0·14	0·38 ± 0·02*
5.0 p.m.	31	3	Trace	0·45 ± 0·11	0·33 ± 0·13

* Difference significant at $P < 0·05$. † Difference significant at $P < 0·01$.

Table 4. *Phosphorylated glycolytic intermediates in rat liver*

Effect of 4 hr. bilateral hind-limb ischaemia (10.0 a.m.–2.0 p.m.) on the phosphorylated glycolytic intermediates in the livers of intact rats given access to food until the beginning of the experiment (fed rats) or fasted for 24 hr. before the experiment (fasted rats). Environmental temperature 18–22°. All results are expressed as μmoles/g. wet wt. (mean ± S.D.).

Results in injured rats are compared with corresponding controls and results in guillotined controls are compared with those in the corresponding controls sampled under sodium pentobarbitone.

Treatment	Time after removal of tourniquets (hr.)	Method of sampling	No. of rats	Glucose 1-phosphate	Hexose 6-phosphate	Fructose 1:6-diphosphate	Phospho-glycerate	α-Glycero-phosphate	β-Glycero-phosphate
						Fed rats			
Control (2.0 p.m.)	—	Pentobarbitone	6	0·47 ± 0·19	0·83 ± 0·25	0·29 ± 0·06	0·18 ± 0·07	1·91 ± 0·25	0·81 ± 0·32
Limb ischaemia	0	Pentobarbitone	5	0·54 ± 0·19	0·71 ± 0·32	0·25 ± 0·07	0·22 ± 0·07	1·76 ± 0·37	0·72 ± 0·25
Control (5.0 p.m.)	—	Pentobarbitone	5	0·53 ± 0·15	0·60 ± 0·12	0·27 ± 0·05	0·18 ± 0·09	2·10 ± 1·07	0·62 ± 0·22
Limb ischaemia	3	Pentobarbitone	6	0·53 ± 0·17	0·56 ± 0·23	0·39 ± 0·06†	0·23 ± 0·08	2·27 ± 1·01	0·99 ± 0·60
Control (5.0 p.m.)	—	Guillotine	6	0·46 ± 0·16	0·95 ± 0·19†	0·16 ± 0·04†	0·21 ± 0·08	0·50 ± 0·24*	0·24 ± 0·17*
Limb ischaemia	3	Guillotine	6	0·41 ± 0·07	0·81 ± 0·42	0·34 ± 0·09†	0·89 ± 0·57†	0·61 ± 0·40	0·41 ± 0·27
Control‡ (5.0 p.m.)	—	Pentobarbitone	8	0·48 ± 0·16	1·15 ± 0·33	0·44 ± 0·14	0·25 ± 0·07	1·61 ± 0·39	0·84 ± 0·19
Limb ischaemia‡	3	Pentobarbitone	9	0·23 ± 0·10†	0·84 ± 0·35	0·46 ± 0·07	0·20 ± 0·07	2·06 ± 0·55	0·98 ± 0·11
						Fasted rats			
Control (2.0 p.m.)	—	Pentobarbitone	4	0·12 ± 0·12	0·46 ± 0·14	0·38 ± 0·02	0·14 ± 0·03	1·82 ± 0·08	0·65 ± 0·06
Limb ischaemia	0	Pentobarbitone	5	0·18 ± 0·05	0·74 ± 0·18*	0·43 ± 0·07*	0·19 ± 0·03*	2·35 ± 0·24†	1·05 ± 0·22*
Control (5.0 p.m.)	—	Pentobarbitone	3	Trace	0·45 ± 0·11	0·33 ± 0·13	0·10 ± 0·06	1·68 ± 0·34	0·68 ± 0·31
Limb ischaemia	3	Pentobarbitone	6	Trace	0·88 ± 0·38	0·57 ± 0·13*	0·22 ± 0·07	1·64 ± 0·79	0·54 ± 0·52

* Difference significant at $P < 0·05$.
† Difference significant at $P < 0·01$.
‡ Cold-acclimatized rats.

in the controls after removal of the tourniquets in the intact but not in the medullectomized rats.

Liver

Method of sampling. Most of the liver samples were obtained under light surgical anaesthesia produced by sodium pentobaribitone but some were quickly excised after guillotining. The results for the control samples obtained by these two methods differed (Tables 4 and 7). The concentrations of fructose 1:6-diphosphate, α-glycerophosphate, β-glycerophosphate and lactate were higher and that of hexose 6-phosphate was lower in the samples taken under anaesthesia. The reason for the change in hexose 6-phosphate is obscure but the other changes would be explained if barbiturate anaesthesia altered the equilibrium between the reduced and oxidized forms of diphosphopyridine nucleotide in favour of the reduced form (Gilmore, 1958), provided that the anaesthesia did not significantly alter the rate of glucose utilization (Stoner *et al.* 1960). Since rats can be guillotined with very little disturbance the values obtained by that method may approximate more closely to those *in vivo*. The concentrations of the phosphorylated glycolytic intermediates in the controls were lower than those reported by LePage (1948) and Derache *et al.* (1956). They agreed more closely with those obtained by ion-exchange chromatography on rabbit liver by Sacks, Kuroda & Hurley (1955).

The concentrations of hexose 6-phosphate, glucose 1-phosphate and fructose 1:6-diphosphate varied with the rat's nutritional state (Table 3), so that the results in injured rats were always compared with those in controls killed at the same time of the day.

Phosphorylated glycolytic intermediates. In fed intact rats the main change was in the fructose 1:6-diphosphate fraction, in which an increase was found with both methods of sampling after the tourniquets had been removed (Table 4). In the samples from guillotined rats there was also an increase in the phosphoglycerate concentration possibly related to the increase in the amount of pyruvate present in these samples (see below). In fasted rats there were more widespread changes at the end of the period of limb ischaemia but 3 hr. later the increase in the fructose 1:6-diphosphate concentration was the only significant change (Table 4).

In control-fed medullectomized rats the concentrations of phosphorylated intermediates were within the range for intact rats. Liver samples, taken under sodium pentobarbitone anaesthesia from four fed medullectomized rats 3 hr. after the 4 hr. period of bilateral hind-limb ischaemia, showed high values for hexose 6-phosphate (1·12 ± 0·38 μmoles/g. wet wt.) and fructose 1:6-diphosphate (0·66 ± 0·15 μmole/g. wet wt.).

Table 5. *Phosphorylated glycolytic intermediates in liver of fed adrenalectomized rats*

Samples were taken under sodium pentobarbitone anaesthesia from control rats between 2.20 and 3.20 p.m., and from rats after a 4 hr. period (10.0 a.m.–2.0 p.m.) of bilateral hind-limb ischaemia at intervals after removal of the tourniquets. Control values are shown as means ± S.D. with number of rats shown in parentheses. Environmental temperature 18–22°.

Treatment	Time after removal of tourniquets (min.)	Fructose 1:6-diphosphate (μmole/g. wet wt.)	Phosphoglycerate (μmole/g. wet wt.)
Controls	—	0·36 ± 0·12 (11)	0·07 ± 0·05 (10)
Limb ischaemia	10	0·29	0·16
	10	0·39	0·15
	15	0·40	0·12
	40	0·24	—
	50	0·41	0·23
	52	0·43	0·15
	65	0·41	0·16
	67	0·59	0·12
	70	0·79	0·10
	72	0·29	0·09
	74	0·39	0·07
	77	0·52	0·10
	80	0·33	0·12

Table 6. *Concentration of phosphorylated glycolytic intermediates in the liver of fed rats at an environmental temperature of 30°*

Experimental rats were subjected to bilateral hind-limb ischaemia between 10.0 a.m. and 2.0 p.m. and were killed under sodium pentobarbitone anaesthesia 15–80 min. later. Number of rats is shown in parentheses.

Treatment	Glucose 1-phosphate	Hexose 6-phosphate	Fructose 1:6-diphosphate	Phosphoglycerate	α-Glycerophosphate	β-Glycerophosphate
	[μmoles/g. wet wt. (mean ± S.D.)]					
Controls	0·46 ± 0·16 (5)	1·67 ± 0·39 (5)	0·32 ± 0·07 (5)	0·16 ± 0·04 (5)	1·61 ± 0·32 (5)	0·86 ± 0·44 (5)
Limb ischaemia	0·29 ± 0·14* (10)	1·19 ± 0·38* (10)	0·33 ± 0·08 (10)	0·13 ± 0·05† (9)	2·21 ± 0·37† (10)	0·82 ± 0·48 (8)

* Difference significant at $P < 0·05$.

† Difference significant at $P < 0·01$.

Table 7. *Lactate, pyruvate and α-oxoglutarate concentrations in rat liver*

Samples were taken from control rats at the times shown and from rats after a 4 hr. period (10.0 a.m.–2.0 p.m.) of bilateral hind-limb ischaemia. Number of rats is shown in parentheses. Environmental temperature 18–20°. Statistical comparison is made between fed controls and between the experimental rats and their corresponding controls.

Treatment	Method of sampling	Pyruvate	Lactate	α-Oxoglutarate	Lactate/pyruvate ratio
		[μmoles/g. wet wt. (mean ± S.D.)]			
		Fed rats			
Control (2.0–5.0 p.m.)	Guillotine	0·033 ± 0·18 (18)	1·06 ± 0·43 (11)	0·015 ± 0·006 (18)	39·6 ± 19·7 (11)
Control (2.0–5.0 p.m.)	Pentobarbitone	0·047 ± 0·19 (8)	3·01 ± 0·88‡ (5)	0·017 ± 0·008 (8)	—
End of limb ischaemia	Guillotine	0·031 ± 0·011 (5)	1·71 ± 0·56* (6)	0·020 ± 0·006 (5)	58·6 ± 10·3 (4)
3 hr. after	Guillotine	0·202 ± 0·120‡ (12)	1·43 ± 0·43 (6)	0·014 ± 0·007 (12)	10·0 ± 7·1† (6)
3 hr. after	Pentobarbitone	0·031 ± 0·013 (10)	2·64 ± 0·55 (7)	0·014 ± 0·01 (10)	—
		Fasted rats			
Control (4.0 p.m.)	Guillotine	0·026 ± 0·009 (3)	0·55 ± 0·31 (3)	0·010 ± 0·002 (3)	22·0 ± 12·0 (3)
2 hr. after	Guillotine	0·154 ± 0·032† (3)	1·72 ± 1·27 (3)	0·015 ± 0·005 (3)	10·8 ± 6·9 (3)
		Fed medullectomized rats			
Control (4.30 p.m.)	Guillotine	0·022 (2)	0·82 (2)	0·008 (2)	36·7 (2)
2·5 hr. after	Guillotine	0·070 ± 0·047 (4)	0·92 ± 0·21 (4)	0·010 ± 0·003 (4)	16·1 ± 6·5 (4)
		Fed adrenalectomized rats			
Control (3.0 p.m.)	Guillotine	0·027 ± 0·011 (6)	0·96 ± 0·32 (6)	0·014 ± 0·003 (6)	39·7 ± 17·8 (6)
1 hr. after	Guillotine	0·074 ± 0·042* (6)	1·11 ± 0·45 (6)	0·015 ± 0·002 (6)	18·7 ± 11·1* (6)

* Difference significant at $P < 0·05$.
‡ Difference significant at $P < 0·001$.
† Difference significant at $P < 0·01$.

Table 8. *Lactate, pyruvate and α-oxoglutarate concentrations in the liver of fed rats at an environmental temperature of 30°*

Samples from control rats were taken at 3.0 p.m. and from rats injured by a 4 hr. period (10.0 a.m.–2.0 p.m.) of bilateral hind-limb ischaemia at intervals after removal of the tourniquets. All rats were guillotined. Control values are shown as means ± S.D. with the number of rats in parentheses.

Treatment	Time after removal of tourniquets (min.)	Pyruvate	Lactate	α-Oxoglutarate	Lactate/pyruvate ratio
		(μmoles/g. wet wt.)			
Control	—	0·029 ± 0·016 (7)	1·38 ± 0·31 (7)	0·018 ± 0·011 (7)	54·3 ± 18·8 (7)
Limb ischaemia	32	0·297	2·05	0·018	6·9
	35	0·131	5·12	0·013	39·0
	59	0·160	2·24	0·014	14·0
	59	0·037	4·70	0·010	126·0
	63	0·075	3·62	0·014	48·3
	70	0·047	4·67	0·009	99·2
	73	0·047	2·15	0·012	46·1
	75	0·042	7·16	0·008	170·0
	77	0·073	3·19	0·014	44·0
	93	0·023	5·27	0·005	231·0
	104	0·028	2·37	0·015	83·7

In cold-acclimatized rats (Table 4) the fructose 1:6-diphosphate concentration was high in the livers of the controls but did not rise any further 3 hr. after the injury.

In adrenalectomized rats samples were taken at different times after removal of the tourniquets (Table 5). The only constant change was an increase, during the early and middle parts of the survival period, in the concentration of phosphoglycerate, which was very low in the controls. Some injured adrenalectomized rats showed high concentrations of fructose 1:6-diphosphate in the liver but the majority did not.

At an environmental temperature of 30° uniform values were obtained between 15 and 80 min. after removal of the tourniquets (Table 6). At no time during this period was there any increase in the concentration of fructose 1:6-diphosphate. Instead the concentrations of glucose 1-phosphate and of hexose 6-phosphate fell and that of α-glycerophosphate rose. The high environmental temperature alone led to a rise in the hexose 6-phosphate concentration in the controls ($P < 0.01$) and even when the level had fallen after injury it was still above that in controls at an air temperature of 18–22°.

Pyruvate and lactate. At the end of the period of limb ischaemia the concentration of lactate in the liver was high in fed intact rats but 3 hr. later the significant changes were a very high pyruvate concentration and a fall in the lactate/pyruvate ratio (Table 7). These changes were found only in samples from guillotined animals. This pattern of a high pyruvate concentration with a low lactate/pyruvate ratio was also found in the liver after limb ischaemia in small groups of medullectomized, adrenalectomized and fasted intact rats (Table 7).

In rats subjected to limb ischaemia at an air temperature of 30° the concentrations of pyruvate and lactate were both high shortly after removal of the tourniquets (Table 8). The change in the pyruvate concentration was more marked and the lactate/pyruvate ratio was low. Later, the pyruvate concentration fell and the lactate/pyruvate ratio rose, reaching very high values when there was a further increase in the pyruvate concentration.

α-Oxoglutarate. The concentration of this compound in both liver and muscle remained unaffected by the experimental procedures (Tables 2, 7 and 8).

DISCUSSION

Changes in injured muscle

The striking changes in the injured hind-limb muscle at the end of the 4 hr. period of ischaemia can be attributed to anaerobic glycolysis. However, glycolysis did not go to completion, for large amounts of hexose monophosphate remained, and the fructose 1:6-diphosphate concentration was very low. This may be due to lack of adenosine triphosphate in the injured muscle (Threlfall & Stoner, 1957). A similar picture was seen after glycolysis in excised muscle by Bailey & Marsh (1952). The large increase in the α-glycerophosphate content of the injured muscle may also be due to the anoxia.

When the tourniquets are removed the muscle is reoxygenated and some adenosine triphosphate can be generated in it (Threlfall & Stoner, 1957), permitting further glycolysis to occur. Some glycogen is also formed in the hind limbs at this time (Stoner, 1958*b*) and the combination of these processes could account for the situation in the damaged muscle 3 hr. later.

Changes in uninjured muscle

The main change in the phosphorylated glycolytic intermediates in the uninjured fore-limb muscle was the increase in the concentration of fructose 1:6-diphosphate after the tourniquets had been removed. The statistically insignificant increases in the hexose monophosphate concentrations probably reflect the active glycogenolysis going on in this muscle before and after removal of the tourniquets (Stoner, 1958*b*). Adrenal-medullectomy prevents this breakdown of muscle glycogen (Stoner, 1958*b*) and then the hexose monophosphate concentrations are well within the control range. Adrenal-medullectomy also prevented the rise in the concentration of fructose 1:6-diphosphate. Cori & Cori (1931) have shown, and we have confirmed (unpublished results), that when muscle glycogenolysis is produced by the injection of adrenaline the concentration of hexose 6-phosphate increases whereas that of fructose 1:6-diphosphate does not. As the formation of the latter will also be increased under these conditions it would appear that an increase in fructose 1:6-diphosphate formation alone does not lead to an observable rise in its concentration. This suggests that the change after injury was due to the partial inhibition of fructose 1:6-diphosphate utilization so that an increase in its concentration was seen only when its rate of formation was also increased.

The absence of an increase in the products of glycolysis in the fore-limb muscle after hind-limb ischaemia in the medullectomized rat suggests that the main reason for the raised muscle-pyruvate concentration in the injured intact rat was its increased rate of formation from glycogen, in the adrenaline-mediated glycogenolysis in the fore-limb muscle after hind-limb ischaemia (Stoner, 1958*b*). If the increase in glycolytic products was due to anoxaemia the main change should have been in the lactate fraction with a rise in the lactate/pyruvate ratio. The absence of a rise in this

ratio is in accord with our previous view that the fore-limb muscle is adequately oxygenated at this time (Threlfall & Stoner, 1957).

Effect of limb ischaemia on the liver

Phosphorylated glycolytic intermediates. The outstanding change in these intermediates was the increase in the concentration of fructose 1:6-diphosphate which occurred in the majority of the groups of injured rats. Since, compared with the change in the intact fed rats, the increase was greater in the medullectomized and fasted rats, which were more sensitive to the injury, and minimal or absent in resistant cold-acclimatized rats, it seemed that the change might be related to the severity of the clinical state. However, this hypothesis of a simple inverse relationship between survival time and the rise in the fructose 1:6-diphosphate concentration could not be sustained because of the findings in the experiments at an environmental temperature of 30°.

Although raising the environmental temperature shortens the survival time after the injury from about 13 hr. to $2{\cdot}22 \pm 0{\cdot}32$ hr. (Stoner & Threlfall, 1960), the behaviour of these rats after injury is so different that it was hardly surprising that they also differed chemically from the other injured rats. The main differences between the behaviour of the injured rats at an air temperature of 30° and those at an air temperature of 18–22° are a rise instead of a fall in liver temperature (Stoner, 1958*a*), a rapid fall in hepatic blood flow instead of a slow fall after a period of some hours during which the flow is within normal limits (Stoner, 1958*a*), a fall in the adenosine triphosphate content of the liver (Stoner & Threlfall, 1954) and more rapid hepatic glycogenolysis (unpublished results). The changes in the phosphorylated glycolytic intermediates found at the higher environmental temperature could well be a reflexion of these events.

The increase in the fructose 1:6-diphosphate concentration found during the first part of the survival period only occurred when the injury caused a fall in liver temperature, i.e. in fed and fasted intact rats and in fed medullectomized rats but not in cold-acclimatized rats, in which there is only a slight fall in liver temperature after limb ischaemia (Stoner, 1958*a*). If the effect is causally related to the temperature change the variable behaviour of the injured adrenalectomized rats requires an explanation, since the temperature regularly falls in these animals.

In the adrenalectomized rat the survival time after 4 hr. bilateral hind-limb ischaemia is only $1{\cdot}86 \pm 0{\cdot}67$ hr. (Stoner & Threlfall, 1960). The contraction of the survival period is largely at the expense of its first part (Stoner, 1960), so that it is difficult to choose a point for comparison with the intact rat. The variability in the short survival time also makes it difficult to know the stage reached by an individual rat. To try to circumvent this difficulty samples were taken at different times after removal of the tourniquets. In some the concentration of fructose 1:6-diphosphate in the liver was high but in the majority it was not. It is not known whether this means that an excess of fructose 1:6-diphosphate is present only for a short time in an individual rat so that its detection is a matter of chance or whether the increase occurs only in rats with accessory adrenal tissue. To minimize the latter possibility the fat around the adrenal was always removed with the gland and there has been no previous indication that such tissue is common in our rats. Other factors may be concerned. Although the results do not exclude a relationship between the fall in temperature and the rise in the fructose 1:6-diphosphate concentration, more work is necessary to elucidate the possible role of the adrenal cortex in this response.

Pyruvate and lactate concentrations. A further significant change in the liver after limb ischaemia was an increase in its pyruvate concentration with a fall in the lactate/pyruvate ratio. When the sampling times were such that the rats were in a comparable state, the picture was common to intact, medullectomized and adrenalectomized rats (Table 7) but was not found when the liver samples were removed under barbiturate anaesthesia. If the excess of pyruvate had been converted into lactate under the influence of sodium pentobarbitone (see above) the change would not have been detected. Reasons have already been given for considering that the results in the guillotined rats represent conditions *in vivo*.

The changes in rats subjected to limb ischaemia at an air temperature of 30° resembled those in other groups more closely than did the changes in the phosphorylated glycolytic intermediates. Some of the difficulties in assessing changes in injured rats at an air temperature of 30° have already been mentioned. In general, the longer the rat has remained alive after the limb ischaemia the shorter its expectation of life, but the survival time is very variable, and, as there is no progressive visible change (unlike in injured rats at the lower environmental temperature), it is extremely difficult to decide an individual rat's proximity to death. The best guide in these rats is the fall in liver blood flow (Stoner, 1958*a*). In the light of our previous work our interpretation of the results in Table 8 is that for a short time after limb ischaemia pyruvate accumulates in the liver as in other injured rats but this process is cut short by hypoxia due to the failing liver blood flow, so that ultimately there is an accumulation of lactate. On this interpretation the rats with high lactate/pyruvate ratios

would be those nearest to death. The large amount of lactate present at all times can be attributed to the enormous breakdown of liver glycogen which occurs under these conditions.

Unlike muscle, the liver contains a relatively large amount of blood, about 0·1 ml./g. wet wt. (Caster, Simon & Armstrong, 1955). From previous studies (Stoner, Threlfall & Green, 1952; Stoner *et al.* 1960) it would seem that in the controls most of the pyruvate could be in the blood of the liver and not in its parenchyma. The majority of the lactate and phosphorylated intermediates, on the other hand, would be in the liver cells (Stoner *et al.* 1952; Bartlett, 1959). Although the blood concentrations of lactate and pyruvate are raised for a time after removal of the tourniquets (McShan, Potter, Goldman, Shipley & Meyer, 1945; Stoner *et al.* 1952, 1960), the levels at the time of sampling could not have accounted for the changes in the liver. This means that the percentage increase in intracellular pyruvate was greater than would be apparent from the values in Tables 7 and 8.

The low lactate/pyruvate ratio in the injured rats is consistent with the view that there is a period of adequate hepatic oxygenation after removal of the tourniquets, although it may be quite short, as in those at an air temperature of 30°.

α-Oxoglutarate concentration. The liver contained more α-oxoglutarate than did muscle. The concentration of this metabolite remained constant throughout the various groups of control and injured rats. Although this result is of interest as liver mitochondria isolated from rats after limb ischaemia behaved in the same way *in vitro* as those from normal rats (Aldridge & Stoner, 1960), the work of Cavallini & Frontali (1954) suggests that most of the α-oxoglutarate could be accounted for by the blood in the liver.

Conclusions

The most significant changes during the early part of the survival period after limb ischaemia in the intact rat were an increase in the concentrations of fructose 1:6-diphosphate and pyruvate in the uninjured muscle and an increase in the concentration of pyruvate in the liver and also of fructose 1:6-diphosphate when its temperature fell. A rise in the concentration of a tissue constituent can be due to its increased formation or decreased utilization or both. In the uninjured muscle, even if the utilization of fructose 1:6-diphosphate and pyruvate was inhibited, the rise in their concentrations was found only when there was over-production by increased glycogenolysis.

The situation in the liver is not so clear. Both fructose 1:6-diphosphate and pyruvate can be formed and utilized by a number of alternative reactions. Previous work with [^{14}C]glucose and [2-^{14}C]pyruvate (Stoner *et al.* 1960) showed that during the early part of the survival period the metabolism of glucose was inhibited in the early stages of its catabolism and also in the steps subsequent to the formation of pyruvate, the oxidation of which was diminished. Those changes were thought to be causally related to the fall in liver temperature. There is agreement between the two sets of experiments in that the present ones also show carbohydrate breakdown to be altered at two such points. Furthermore it would seem that the two changes can arise independently, as in the rats subjected to limb ischaemia at an air temperature of 30° where there was a rise in the pyruvate concentration but not in that of fructose 1:6-diphosphate. The latter increase was only seen when the liver temperature fell. Although the mechanism of these changes cannot yet be described some possibilities can be eliminated.

Increased formation of fructose 1:6-diphosphate and pyruvate could occur by: (1) an increase in glucose breakdown; (2) an increase in glycogen breakdown in the liver; (3) increased gluconeogenesis from the extra amounts of pyruvate and lactate being brought to the liver after the increase in muscle glycogenolysis; (4) gluconeogenesis from non-carbohydrate sources.

The first of these possibilities can be excluded (Stoner *et al.* 1960). Neither (2) nor (3) is likely to be a major factor because the enormous differences in the amounts of liver and muscle glycogen broken down in the different experimental groups (Stoner, 1958*b*) were not reflected in the results. The influence of (4) is difficult to assess, but in accounting for the alterations in the distribution of glucose and glycogen after limb ischaemia (Stoner, 1958*b*) it was not necessary to postulate any increase in gluconeogenesis from non-carbohydrate sources above the amount in comparable controls.

An acceptable explanation for the increased concentrations of fructose 1:6-diphosphate and pyruvate in the liver would be a decrease in their utilization. In the breakdown of fructose 1:6-diphosphate towards pyruvate the most important step would probably be the triose phosphate-dehydrogenase reaction. Because of the ready reversibility of the aldolase reaction, inhibition of the triose phosphate-dehydrogenase reaction leads to fructose 1:6-diphosphate accumulation (see Krebs & Kornberg, 1957). Similarly, if the oxidation of pyruvate is decreased its concentration will rise unless there is a compensatory increase in the other reactions utilizing pyruvate.

The most important feature of these results is the way in which they confirm, by quite different methods, the conclusion of the previous paper (Stoner *et al.* 1960) that the carbohydrate

metabolism of these injured rats is altered at two points, and the help they give in planning further experiments to elucidate the altered mechanisms.

SUMMARY

1. The early effects of bilateral hind-limb ischaemia on the concentrations of phosphorylated glycolytic intermediates, pyruvate, lactate and α-oxoglutarate, in muscle and liver have been investigated in intact, intact cold-acclimatized, adrenal-medullectomized and adrenalectomized rats at air temperatures between 18° and 22° and also in intact rats at an air temperature of 30°.

2. The changes in the phosphorylated glycolytic intermediates in the injured muscle during ischaemia were the same as those reported for anoxic muscle *in vitro*.

3. In the uninjured fore-limb muscle the fructose 1:6-diphosphate and pyruvate concentrations rose after hind-limb ischaemia. These changes were attributed to increased glycogenolysis and to decreased utilization of fructose 1:6-diphosphate and possibly also of pyruvate.

4. The most constant change in the liver after limb ischaemia was an increase in the pyruvate concentration and also in that of fructose 1:6-diphosphate when conditions were such that the liver temperature fell. These changes are attributed to a decrease in the utilization of fructose 1:6-diphosphate and in the oxidation of pyruvate.

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The Degradation of Cotton Cellulose by the Extracellular Cellulase of *Myrothecium verrucaria*

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Many papers on the action of extracts from cellulolytic fungi on various cellulosic substrates have been published over the last two decades. It is now well established that any pretreatment of cellulose involving either swelling or dissolution increases its accessibility both to attack by cellulase and to other reagents. This aspect of the subject has been adequately reviewed elsewhere (Pigman, 1951; Cumberbirch, 1955). It is still uncertain, however, whether the attack of the enzyme on the accessible glucoside linkages in the cellulose is random or endwise, as it is known to be with certain other glucosidases. An answer can only be obtained by examining the relation between such factors as the

change in degree of polymerization, the loss of weight and the number of end groups produced during hydrolysis.

Whitaker (1957) has investigated the hydrolytic action of the cellulase of *Myrothecium verrucaria* on a cellulose that had previously been swollen and partially degraded by treatment with 15N-phosphoric acid and has shown that the changes of both weight- and number-average degree of polymerization in relation to the loss of weight during the hydrolysis are consistent with a theory of random attack on all the β-glucoside linkages. It is well known, however, that the degradation of cotton cellulose by fungi produces very much smaller changes in degree of polymerization (as measured by the fluidity of a standard solution of the cellulose in cuprammonium), for a given loss of strength, than does acid degradation (Searle, 1929; Fargher, 1953). Although this difference has been attributed to the highly localized growth of the fungus, which causes whole regions of the substrate to be digested away and leaves others untouched, Walseth (1952), working with a cellulase from *Aspergillus niger*, has suggested that it is due to the limited accessibility of the substrate to attack by the enzyme. The theory of random attack proposed by Whitaker will then apply only to a cellulose substrate sufficiently swollen to be more than usually accessible to large molecules. The experiments described in this paper have shown that the degradation of cotton cellulose, even when it has been swollen by pretreatment with sodium hydroxide solution (mercerized), by the extracellular extract of *M. verrucaria* is neither random nor endwise, and an alternative mechanism is suggested.

EXPERIMENTAL

Organism. The organism was *Myrothecium verrucaria*, obtained as strain 45,541 from the Commonwealth Mycological Institute (originally Q. M. 460 isolated by M. H. Downing from a cotton bale, Washington, D.C., U.S.A.).

Enzyme preparations. The methods of Whitaker (1953) were followed as far as the fractionation with ethanol to provide a partially purified cellulase preparation. This was preserved by freeze-drying and storage at 0°. No loss of activity towards carboxymethylcellulose could be detected after several months. The organism was also grown on a smaller scale in the same medium (300 ml. batches in 1 l. shake flasks) to provide simple culture filtrates which were used without purification.

Cellulose substrates. (*a*) Cotton yarn. A Sudan Sakel yarn of count 20 (0·295 mg./cm.) was given two successive scouring treatments by heating in an autoclave with 1% sodium hydroxide solution at 140° for 3 hr. It had a viscosity-average degree of polymerization ($\bar{P}_V$) of approximately 6000, calculated from the fluidity (3·5 P^{-1}) of a standard (0·5%, w/v) solution in standard cuprammonium.

(*b*) Cotton sliver. A scoured Texas sliver with approx. $\bar{P}_V$ 6500 (fluidity of 0·5% solution in cuprammonium, 2·6 P^{-1}).

(*c*) Cotton flock (supplied as C/8 flock by Hutchinson Ltd., Ramsey Mill, Chadderton, Oldham, Lancs.). This was scoured, mechanically powdered flock having approx. $\bar{P}_V$ 2900 (fluidity of 0·5% solution in cuprammonium, 8·3 P^{-1}).

Mercerization. The cotton was swollen by immersion without tension in 25% sodium hydroxide solution at 2° for 4 hr. After removal from the alkali the sample was washed until the wash-water remained neutral and stored at 2° under water to keep it swollen.

Assay of the activity of the enzyme towards carboxymethylcellulose. To a solution of carboxymethylcellulose (Cellofas B, Imperial Chemical Industries Ltd.; 200 mg. in 10 ml. of 0·1M-acetate buffer, pH 5·6) were added water (9 ml.) and a solution of crystalline bovine plasma albumin (300 μg. in 1 ml. of water) (Whitaker, 1953). The solution was filtered through a sintered-glass disk and brought to 30° in a thermostat. An appropriate volume of the cellulase preparation was measured from a micrometer pipette into a small glass vessel. The volume was made up where necessary to approximately 0·5 ml. with water and the vessel was dropped into the carboxymethylcellulose solution. The two solutions were mixed and quickly introduced into an Ostwald viscometer, which was already in position in the thermostat. Measurements of viscosity were made after known degradation times, zero time being taken as the time at which the enzyme was added to the substrate. Values of specific viscosity ($\eta_{sp.} = \eta_{rel.} - 1$) were calculated. The reciprocals of the specific viscosities were plotted against the times of degradation in minutes (cf. Thomas, 1956). The slope of the line so generated was found to be proportional to the enzyme concentration over the experimentally convenient range of concentrations. The numerical value of this slope (min.$^{-1}$), calculated for a concentration of original enzyme preparation of 0·500 ml./20 ml. of substrate, was chosen as a measure of the activity of the preparation (carboxymethylcellulase units). Since the enzymic degradation was continuing during the measurement, the viscosity of the solution was falling continuously. It was assumed that the fluidity of the solution varied linearly with time over the small range involved during the making of any one measurement, and hence the time of degradation was taken as half-way between the start and finish of the flow. This was shown not to involve any serious error when the rate of change of fluidity was of the order normally found. The specific viscosity of a solution of carboxymethylcellulose, prepared as described above, but with the addition of the same quantity of enzyme inhibited with mercuric acetate, was 4·05.

Enzymic degradation of cellulose. The cotton sample (1·5 g.) was placed in a 250 ml. conical flask, to which was then added sodium acetate buffer (0·1M; pH 5·6; 60 ml.) containing an antiseptic (sodium pentachlorophenate, 0·05%), crystalline bovine plasma albumin (5 ml. of a solution containing 300 μg./ml.), water (25 ml.) and finally the appropriate enzyme solution, contained in 10 ml. of water. The samples were placed on a rotary shaking machine at 30° for the required time. To check the sterility of the preparation a sample of the liquor was then used to inoculate a slope of oatmeal–agar. The loss of weight was determined by filtering off the remaining insoluble material, which was washed and dried over phosphorus pentoxide to

constant weight in a vacuum oven at 60°. The fluidity of a standard solution in cuprammonium was measured on a sample (300 mg.) taken from each residue and a further sample (approx. 1 g.) was used for the determination of the loss of weight on heating with alkali.

Degradation of cellulose by hydrochloric acid. The cotton yarn [substrate (*a*) above; 1 g.] was immersed in 6N-hydrochloric acid (30 ml.) at 30° for from 2 to 240 hr. with occasional mechanical shaking. The acid was removed by filtration, the residual cellulosic material was washed with water at room temperature until the washings were acid-free, and dried to constant weight in a vacuum oven at 60° over phosphorus pentoxide and the loss of weight was calculated. The fluidity of a standard solution of the residue in cuprammonium, and the loss of weight on heating with alkali, were measured.

Determination of the viscosity-average degree of polymerization ($\bar{P}_v$) of cellulose. The fluidity (P^{-1}) of a standard (0·5%, w/v) solution of the cellulose in standard cuprammonium (containing 15 g. of copper/l., 200 g. of ammonia/l.) at 20° was measured by the method of Calvert & Clibbens (1951). The intrinsic viscosity (i) was obtained from table VIII given by these authors. $\bar{P}_v$ (as defined by Flory, 1953) was calculated from the intrinsic viscosity by means of the relation:

$$i = 0{\cdot}0319 . \bar{P}_v^{0{\cdot}657}$$

derived by Cumberbirch & Harland (1958). This relation is based on experimental data for a range of $\bar{P}_v$, 190–2000, and therefore the values above 2000 are only approximate. This approximation is justified, since lack of accuracy in these figures does not affect the argument presented here.

Determination of loss of weight on heating with alkali. The loss of weight on heating with alkali (Clibbens, Geake & Ridge, 1927) was used as an empirical measure of the terminal aldehyde groups in the cellulose samples. An accurately weighed amount of the dried sample of cellulose or modified cellulose (approx. 1 g.) was placed in a 150 ml. conical flask of Pyrex glass, which was then nearly filled with cold 1% sodium hydroxide solution. The flask was closed with a pear-bulb stopper to exclude air and heated in a boiling-water bath for 6 hr. The undissolved residue was collected in a sintered-glass crucible of porosity 3, washed, dried in a vacuum over phosphorus pentoxide at 60° and weighed. The percentage weight loss was calculated and is referred to below as the loss of weight on heating with alkali.

RESULTS

Degradation of cellulose. Degradation of the various substrates already described at enzyme activities ranging from $0{\cdot}4 \times 10^{-2}$ to 50×10^{-2} carboxy-

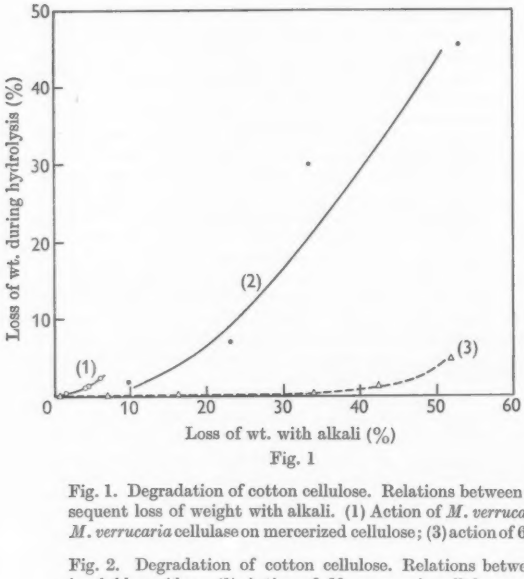

Fig. 1

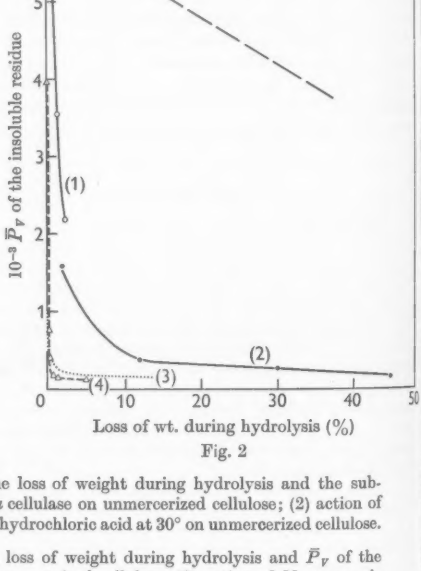

Fig. 2

Fig. 1. Degradation of cotton cellulose. Relations between the loss of weight during hydrolysis and the subsequent loss of weight with alkali. (1) Action of *M. verrucaria* cellulase on unmercerized cellulose; (2) action of *M. verrucaria* cellulase on mercerized cellulose; (3) action of 6N-hydrochloric acid at 30° on unmercerized cellulose.

Fig. 2. Degradation of cotton cellulose. Relations between loss of weight during hydrolysis and $\bar{P}_v$ of the insoluble residue. (1) Action of *M. verrucaria* cellulase on unmercerized cellulose; (2) action of *M. verrucaria* cellulase on mercerized cellulose; (3) action of *M. verrucaria* cellulase on cellulose swollen with phosphoric acid [interpolated from results given by Whitaker (1957)]; (4) action of 6N-hydrochloric acid at 30° on unmercerized cellulose. The broken line at the top of the graph is the theoretical curve for endwise attack.

methylcellulase units, and for various times, yielded data which are presented in Figs. 1 and 2. The relation between the loss of weight during enzymolysis and the subsequent loss on heating with alkali is shown in Fig. 1. Fig. 2 relates the loss during enzymolysis to the $\bar{P}_v$ of the insoluble residue. It was not possible to cause more than 2·5% loss of weight by enzymic hydrolysis of the unswollen celluloses and the range of the experiments was extended by using cellulose substrates which had been made more accessible by mercerization. With these materials weight losses of up to 45% were obtained. The results obtained at the different times and different enzyme activities (with both crude and partially purified preparations) lie on the same curves, as do those with the different substrates, except for those from the mercerized samples, which lie on curves that can be distinguished from those from the unmercerized samples in both Figures.

Results for the acid degradation of unmercerized cotton cellulose and those obtained by Whitaker (1957) for the enzymic degradation of his swollen cellulose are given for comparison. Whitaker used the relation $\bar{P}_W = 107i$ (where $\bar{P}_W$ = weight-average degree of polymerization) to calculate, from the viscosity of the derived nitrate in ethyl acetate solution, values of degree of polymerization which are plotted here as values of $\bar{P}_v$.

DISCUSSION

Since there is no satisfactory absolute method for the determination of terminal aldehyde groups in modified celluloses the loss of weight on heating with alkali under standardized conditions, which has been shown to have the same analytical significance as copper reducing power (Clibbens *et al.* 1927), has been used as an empirical measure of the content of these groups. If, as is deduced below, enzymic attack occurs only in the most accessible regions of cellulose it seems probable that the aldehyde groups released will be at least as accessible to degradation by alkali as those from acid attack. If a difference in accessibility to alkali exists it will therefore cause underestimation of the content of terminal aldehyde groups in the acid-hydrolysed celluloses. The contrast between acid and enzymic attack may therefore be greater than the results (Fig. 1) show. It is clear from Fig. 1 that, for the production of a given number of terminal aldehyde groups in the insoluble residue, considerably more substrate is removed during enzymic than during acid attack. Hence, whether or not the acid hydrolysis of cellulose is a random process with respect to all the β-glucoside linkages, the enzymic hydrolysis cannot be. This evidence, taken alone, would suggest predominantly endwise attack. However, reference to Fig. 2 shows that the attack is not endwise. For endwise attack the relation between the amount of weight lost on hydrolysis and the $\bar{P}_v$ of the residue is represented approximately by the straight line, which was calculated on the assumption of a uniform initial chain length. For a given loss of weight the effect on $\bar{P}_v$ produced by the enzymic degradation is clearly much greater than that corresponding to endwise attack. The relation between the weight loss during hydrolysis and the $\bar{P}_v$ of the insoluble residue found by Whitaker (curve 3; Fig. 2) is very similar to that found for material degraded by acid (curve 4; Fig. 2) and is markedly different from that for either unmercerized or even mercerized cellulose after attack by cellulase.

The mode of action of cellulase on an unswollen cellulose suggested by these experiments is one of initial attack at relatively few sites along the length of a molecule. This is then followed by cleavage of a number of linkages in the immediate vicinity of these sites. The material removed at this stage is converted into soluble carbohydrates. The reason for this restricted attack is not apparent until one considers the cellulose molecule as a component of the cotton hair. Cotton cellulose is known to contain regions of varying accessibility and it is suggested that since *M. verrucaria* cellulase has a large molecule (Whitaker, Colvin & Cook, 1954) it is probably incapable of penetrating any but the regions of highest accessibility, and will also have little mobility within the cellulose structure. When the cotton has been swollen by treatment with alkali it is more generally accessible, but the attack of the enzyme, although much more extensive, is still far from random, presumably because the enzyme molecules still hydrolyse preferentially linkages in the immediate vicinity of their initial points of attack. If, however, the cellulose is swollen in phosphoric acid, or if the molecular size of the catalyst is smaller (for example, if an acid is used in place of an enzyme), attack of a more random nature is observed. This is presumably due to the increased mobility of the catalyst molecule within the substrate.

SUMMARY

1. Extracellular extracts of *Myrothecium verrucaria* have been used to study the mechanism of cellulase action on unmercerized and mercerized cotton cellulose.

2. Relations between loss of weight during hydrolysis and (1) decrease of viscosity-average degree of polymerization and (2) the further loss of weight on heating with alkali have been determined and compared with results similarly obtained by (*a*) acid degradation of unmercerized cellulose and (*b*) enzymic degradation of swollen cellulose.

3. These comparisons suggest that the enzyme, being composed of large molecules with restricted mobility in the substrate, removes a number of adjacent glucose residues from each of relatively few sites of attack.

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Folic Acid and the Synthesis of Methionine by Extracts of *Escherichia coli*

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The synthesis of methionine from homocysteine and serine by enzymic extracts of *Escherichia coli* has been found in earlier work from this Laboratory to require a cofactor of the folic acid group. In experiments with dialysed and Dowex 1-treated enzymic extracts, the folic acid cofactor can be supplied either by an extract of heated *E. coli*, or under certain conditions by tetrahydropteroylglutamate (Szulmajster & Woods, 1960; Kisliuk & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960). These two sources of cofactor differ in their properties, and in the conditions under which they show activity with extracts of *E. coli* PA15, a glycine or serine auxotroph. First, the cofactor activity of tetrahydropteroylglutamate is rapidly lost on exposure to air, whereas that of the extract of heated *E. coli* is stable under these conditions. Secondly, the utilization of tetrahydropteroylglutamate by extracts of strain PA15 is dependent upon cobalamin. With ultrasonic extracts the addition of cobalamin to the reaction mixtures is sufficient to allow tetrahydropteroylglutamate to act as cofactor (Guest *et al.* 1960), whereas with extracts of acetone-dried organisms it is necessary that the organisms should have been grown in the presence of cobalamin for tetrahydropteroylglutamate to be effective (Kisliuk & Woods, 1960). On the other hand, when extract of heated *E. coli* is used as the source of cofactor, cobalamin is not needed with either type of enzymic extract. Thirdly, the methionine synthesis promoted by extract of heated *E. coli* is inhibited by tetrahydropteroylglutamate in the absence of cobalamin (Kisliuk & Woods, 1960; Guest *et al.* 1960).

E. coli 121/176 is an auxotroph requiring cobalamin or methionine for growth. Ultrasonic extracts prepared from organisms grown in the presence of methionine differ from those of strain PA15 in that cobalamin is needed for methionine synthesis promoted by either extract of heated *E. coli* or by tetrahydropteroylglutamate.

The observed differences between extract of heated *E. coli* and tetrahydropteroylglutamate in this reaction suggest that the latter is not identical with the factor or factors present in the extract of heated *E. coli*. Although most work with reactions requiring folic acid cofactors has been done with tetrahydropteroylglutamate, experiments with two other such reactions have also shown that this compound is not identical with the active substance or substances in extracts of heated organisms.

Wright (1958) has found seven compounds, all pteridine derivatives, in heated extracts of *Clostridium cylindrosporum* which act as cofactors for the

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conversion of serine into glycine by enzyme-containing extracts of *Clostridium sticklandii*. The common requirement for cofactor activity was the presence of three or seven glutamic acid residues, regardless of whether the pteridine part of the molecule was reduced or unreduced, formylated or unformylated. Two of these compounds have been identified as pteroyltriglutamate and N^{10}-formylpteroyltriglutamate. The others are believed to be derivatives of N^5-formyltetrahydropteroyltriglutamate and N^5-formyltetrahydropteroylheptaglutamate, each containing additional glycine residues. The remaining compounds are more complex and as yet uncharacterized.

The cofactor activity of heated extracts of *Clostridium acidi-urici* in the decomposition of formimidoylglycine (formiminoglycine) by enzyme-containing extracts of the same organism (Rabinowitz, 1959, and personal communication) is vested entirely in tetrahydropteroyltriglutamic acid. No other active compounds were found.

These findings are in accordance with the general observation that naturally occurring forms of folic acid, whether or not they show cofactor activity for any of these reactions, are very largely triglutamates and heptaglutamates (Wright, 1958; Rabinowitz, 1960). Little, if any, of the monoglutamate forms are found in fresh tissues, though they are formed rapidly from the conjugated compounds during autolysis.

These results suggest that the folic acid components of the extract of heated *E. coli* which are active as cofactors of methionine synthesis may be polyglutamyl compounds. In the present work, the cofactor activities of various synthetic polyglutamates of the folic acid group have been investigated and their properties compared with those of tetrahydropteroylglutamate and the extract of heated *E. coli*. A brief report of part of this work has already appeared (Guest & Jones, 1960).

Throughout this paper the term folic acid will be used in general reference to all members of this group of vitamins and growth factors.

MATERIALS AND METHODS

Organisms. Stock cultures of strains of *E. coli* were maintained on slopes of tryptic meat–agar, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°. Most of the work was done with *E. coli* strain PA15, an auxotroph which requires either serine or glycine for growth and was originally obtained from Dr Barbara Wright. *E. coli* strain 121/176, a methionine or cobalamin auxotroph (Davis & Mingioli, 1950), was used in a few experiments; it was obtained from Professor B. D. Davis.

Media and growth. The organisms were grown on solid media in enamelled trays. The media and methods of growth of organisms for the subsequent preparation of ultrasonic and heated extracts have been described by Guest *et al.* (1960), and for the preparation of acetone-dried powders by Szulmajster & Woods (1960). In those experiments where cobalamin (13 μm-moles/500 ml. of medium) was present during growth, it was added with the inoculum to the surface of each tray.

Preparation of enzyme-containing extracts. Ultrasonic extracts were prepared by the method of Guest *et al.* (1960). Potassium phosphate buffer (0·05 M, pH 7·8) was used throughout for the preparation of suspensions of organisms before ultrasonic treatment and for the dialysis of the extracts.

Extracts of acetone-dried organisms were prepared in general as described by Guest & Woods (1960). The acetone-dried powders, homogenized in water, were, however, subjected to ultrasonic vibration (25 kcyc./sec.; 2·5 min.; 600 w with an ultrasonic generator type E7590B of Mullard Ltd., London, W.C. 1) before being centrifuged and dialysed. Compared with extracts made by homogenization only, such treatment increased the total activity extracted per gram of powder about 2·5-fold with only a small decrease in specific activity.

All extracts were treated before use with Dowex 1X8 resin (chloride form; 100–200 mesh) by the method of Kisliuk & Woods (1960). The protein concentration of extracts was estimated spectrophotometrically by the method of Layne (1957).

Extract of heated Escherichia coli. This was prepared as described by Guest *et al.* (1960) and contained 14 mg. dry wt. of solid material/ml.

Test for methionine synthesis. The basal reaction mixture used in experiments with ultrasonic extracts contained (μmoles in a final volume of 2 ml.): potassium phosphate buffer, pH 7·8, 250; DL-homocysteine, 10; L-serine, 10; hexose diphosphate, 15 or glucose, 20; $MgCl_2$, 10; adenosine triphosphate (ATP), 10; diphosphopyridine nucleotide (DPN), 1; pyridoxal phosphate, 0·5. In experiments with extracts of acetone-dried organisms DL-homocysteine was increased to 20 μmoles and pyridoxal phosphate to 1 μmole. The amount of enzyme-containing extract used contained 10 mg. of protein.

Reaction mixtures were incubated at 37° for 2–4 hr. in an atmosphere of H_2. After heating at 100° for 3 min. the reaction mixtures were centrifuged and the supernatant fluids used for the estimation of methionine.

Methionine was assayed microbiologically with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 by the method described by Gibson & Woods (1960). DL-Methionine was used as standard; the organism responds only to the L-isomer.

Estimation of folic acid. Microbiological assays with *Lactobacillus casei* (A.T.C.C. no. 7469), *Streptococcus faecalis* (A.T.C.C. no. 8043) and *Pediococcus cerevisiae* (*Leuconostoc citrovorum*) (A.T.C.C. no. 8081) were employed for the estimation of various forms of folic acid. *Lactobacillus casei* grows with mono-, di- and tri-glutamate forms of folic acid but not with pteroate; *S. faecalis* responds to pteroate, pteroylglutamate and their derivatives but not to di- and tri-glutamate; *P. cerevisiae*, however, requires a reduced form of folic acid, showing no response with any unreduced compound. Under the conditions of the assay only reduced compounds substituted in the N^5-position would be expected to be stable. The response of *P. cerevisiae* to reduced triglutamate compounds is uncertain (Rabinowitz, 1960). None of the organisms responds to pteroylheptaglutamate.

The three organisms were maintained on medium B1 of Nimmo-Smith, Lascelles & Woods (1948). Stab cultures were transferred fortnightly, incubated for 16 hr. at 37° and stored at 4°.

A single basal medium, based upon that of Toennies, Frank & Gallant (1952), was used for all three organisms. It contained (in each litre of final medium): tryptic digest of casein, 500 ml.; DL-alanine, 200 mg.; L-asparagine, 100 mg., L-cysteine, 200 mg.; glucose, 20 g.; KH_2PO_4, 0·1 g.; $MgSO_4,7H_2O$, 200 mg.; NaCl, 10 mg.; $FeSO_4,7H_2O$, 10 mg.; $MnSO_4,7H_2O$, 10 mg.; adenine, 10 mg.; guanine, 10 mg.; xanthine, 10 mg.; uracil, 10 mg.; nicotinic acid, 0·5 mg.; calcium pantothenate, 0·5 mg.; thiamine hydrochloride, 0·5 mg.; riboflavin, 0·5 mg.; pyridoxamine hydrochloride, 0·2 mg.; *p*-aminobenzoic acid, 0·1 mg.; biotin, 10 μg.; pH adjusted to 6·4–6·5. The tryptic digest of casein was prepared as described by Roberts & Snell (1946) and modified by Nimmo-Smith *et al.* (1948). The medium was normally made up with the quantities above in a volume of 700 ml., and then diluted to the correct strength during the preparation of the assays.

The strong basal medium (3·5 ml.), test samples and water to a total volume of 4·9 ml. were distributed in 125 mm. × 16 mm. tubes. Standards containing pteroylglutamic acid, pteroyltriglutamic acid or leucovorin (synthetic N^5-formyltetrahydropteroylglutamic acid) (0·05–0·5 μmM) were included in each assay. Leucovorin is a mixture of two isomers, only one of which supports growth of the assay organisms. The tubes were autoclaved for 2·5 min. at 121°. The inoculum was derived from a culture grown in medium B2 of Nimmo-Smith *et al.* (1948) for 16 hr. at 37°, harvested and washed with water. Inoculum development tubes, containing 5 ml. of basal medium supplemented with pteroylglutamic acid or leucovorin (0·3 μmM), were inoculated with an amount of washed organisms equivalent to about 0·1 mg. dry wt. and incubated for 4–6 hr. at 37°, by which time the cultures were in the logarithmic phase of growth. This culture was centrifuged and the organisms were suspended in the culture volume of water. A tenfold dilution of this suspension was used as the inoculum for the assays (0·1 ml./tube).

Tubes were incubated upright in air for 20–22 hr. at 37°. Growth was assessed in a Hilger Spekker Photoelectric Absorptiometer fitted with a neutral filter (H 508). The uninoculated medium was used as a blank.

The method used to degrade polyglutamates of the folic acid group to the corresponding monoglutamates was that recommended by the Association of Vitamin Chemists Inc. (1951) with a conjugase preparation from hog kidney. The final filtrate, containing about 4 μm-moles of folic acid/ml., was adjusted to pH 7, suitably diluted, and used for assay with the various organisms. The efficacy of the conjugase treatment was checked by a control containing a known amount of pteroyltriglutamate. The results were corrected for the folic acid content of the hog-kidney extract (about 25 $\mu\mu$moles/ml.).

Chemicals. Pteroyl-γ-glutamyl-γ-glutamylglutamic acid (generally referred to as pteroyltriglutamate) was a gift from Dr E. L. R. Stokstad. For some experiments it was purified by chromatography on a column of cellulose powder with 0·1 M-sodium phosphate buffer, pH 7·0, saturated with *iso*amyl alcohol at 25°, as solvent. The purified material was precipitated several times from neutral solution with acid and finally freeze-dried.

N^{10}-Formylpteroyltriglutamate was prepared by dissolving pteroyltriglutamate (100 mg.) in 4 ml. of 98% formic acid and keeping it in the dark for 48 hr. The formic acid was then removed by freeze-drying, and the product dissolved in water and again freeze-dried. This gave a yellow material, which was purified by repeated solution in water and precipitation with acid.

A solution of pteroylheptaglutamic acid was obtained as a gift from Dr E. Kodicek. Its purity was checked by microbiological assay. The amount of active material was only about half that originally present in the solution but 95% of this was inactive for both *S. faecalis* R and *Lactobacillus casei* before conjugase treatment and active for both organisms after treatment with conjugase.

Tetrahydropteroylglutamic acid was prepared from 0·5 or 1·0 g. of pteroylglutamic acid by the method of Kisliuk & Woods (1960) and stored as the solid under H_2.

Tetrahydropteroyltriglutamic acid and N^{10}-formyltetrahydropteroyltriglutamic acid were prepared as solutions immediately before use by the following method. Platinic oxide catalyst (Adam's catalyst) (2–5 mg.) was suspended in 0·02 M-sodium phosphate buffer, pH 7·0, in the main vessel of a Warburg manometer. Pteroyltriglutamic acid or N^{10}-formylpteroyltriglutamic acid (4–16 μmoles), dissolved in 0·02 M-sodium phosphate buffer and adjusted to pH 7·0, was placed in the side arm. The manometer vessel, protected from light with a bag of black material, was filled with H_2 and shaken in a water bath at 37°. The uptake of H_2 by the catalyst was allowed to proceed to completion (usually 30–40 min.). The folic acid compound was then added and the mixture again shaken at 37° until an amount of H_2 corresponding to 2 moles of H_2/mole of the folic acid compound had been taken up (30–90 min.). The solution of reduced pteridine (1–5 mM) was withdrawn by a Pasteur pipette with a plug of cotton wool in its tip to filter off the suspended catalyst. The solution was then immediately added to the reaction mixtures for testing methionine synthesis.

N^5-Formyltetrahydropteroyltriglutamate was prepared by a modification of the method used for the synthesis of the corresponding monoglutamate by Shive, Bardos, Bond & Rogers (1950) and Ericson (1953). A solution of N^{10}-formyltetrahydropteroyltriglutamate was prepared as described above and 2-mercaptoethanol (final concentration 0·4 M) added. The solution was adjusted to pH 8·2 and autoclaved for 1 hr. at 121°. After cooling and neutralizing, the solution contained N^5-formyltetrahydropteroyltriglutamate in 20–40% yield. The compound was purified either by chromatography on triethylaminoethylcellulose with a gradient from water to 0·5 M-potassium phosphate buffer, pH 6·1, or by chromatographing on Whatman no. 3 paper, with, as solvent, 0·24 M-citric acid brought to pH 9·0 with ammonia and saturated with *iso*amyl alcohol at 25°. The amount of N^5-formyltetrahydropteroyltriglutamic acid in the product was estimated (*a*) by microbiological assay with *P. cerevisiae* before and after conjugase treatment and (*b*) by the increase in absorption at 350 mμ when the solution was adjusted to pH 1·0 (Rabinowitz, 1960).

Tetrahydropteroylheptaglutamic acid was prepared by adjusting the solution of pteroylheptaglutamic acid (containing 5 μm-moles) to 0·02 M and pH 7·0 with sodium phosphate buffer, adding 2 mg. of platinic oxide catalyst, and shaking for 1 hr. at room temperature in an atmosphere of H_2. The amount of material available was too small to allow the H_2 uptake to be measured manometrically.

Cobalamin was crystalline material obtained from British Drug Houses Ltd., Poole, Dorset; it was about 90% pure by spectrophotometric assay. ATP and DPN were products of Sigma Chemical Co., St Louis, Mo., U.S.A. The DL-homocysteine was from either Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. or Mann Research Laboratories Inc., New York, N.Y., U.S.A.

RESULTS

Methionine synthesis by ultrasonic extracts of Escherichia coli *PA 15*

Extract of heated Escherichia coli. With dialysed and Dowex 1-treated ultrasonic extracts of *E. coli* PA 15, extract of heated organisms is able to act as the source of folic acid cofactor for methionine synthesis and its activity is unaffected by added cobalamin (Guest *et al.* 1960).

The folic acid content of a freshly prepared batch of extract of heated organisms has been measured by microbiological assay with the three test organisms both before and after treatment with a conjugase preparation (Table 1). Much the greatest part of the folic acid was in the form of polyglutamates, since a very large increase in activity for both *S. faecalis* and *P. cerevisiae* was observed on conjugase treatment. The increase in activity for *Lactobacillus casei* on conjugase treatment suggests that a portion of the polyglutamates contain more than three glutamic acid residues.

With a sample of extract of heated organisms of known folic acid content, the amount of this extract required for half-maximum methionine synthesis was determined. The folic acid concentration of a reaction mixture containing this amount of extract of heated organisms is given in Table 1. The values were determined with the three assay organisms after conjugase treatment of the heated extract.

The cofactor activity of extract of heated organisms is inhibited by tetrahydropteroylglutamate (Kisliuk & Woods, 1960; Guest *et al.* 1960). This inhibition has now been examined quantitatively by the method of Lineweaver & Burk (1934) and was found to be competitive (Fig. 1). The K_i for etrtahydropteroylglutamate was 2·2 μM.

Tetrahydropteroylglutamate. This compound is only active as a cofactor of methionine synthesis by ultrasonic extracts in the presence of added cobalamin (Guest *et al.* 1960). The K_m for tetrahydropteroylglutamate in the presence of 70 $\mu\mu$moles of cobalamin was 180 μM.

Tetrahydropteroyltriglutamate. In contrast with the monoglutamate, this substance showed cofactor activity in the absence of added cobalamin and its activity was not appreciably affected by the addition of cobalamin (70 $\mu\mu$moles) to the reaction mixtures (Fig. 2). The K_m for tetrahydropteroyl-

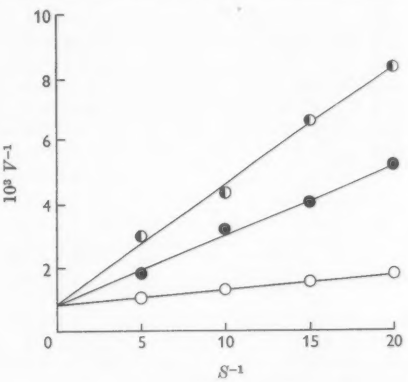

Fig. 1. Effect of tetrahydropteroylglutamate on methionine synthesis by ultrasonic extracts of *E. coli* PA 15 with extract of heated organisms as cofactor. Incubation time, 2 hr. *S*, amount of extract of heated organisms (ml.); *V*, methionine formation (μm-moles of L-methionine/mg. of protein). Concentration of tetrahydropteroylglutamate: ○, nil; ●, 10 μM; ◐, 20 μM.

Table 1. *Folic acid content of extract of heated* Escherichia coli

A freshly prepared sample of extract of heated *E. coli* was used. Conjugase treatment, microbiological assays and tests of the synthesis of methionine were performed as described in the Methods section. The concentration of heated extract in the reaction mixture required for half-maximal methionine synthesis was determined by the method of Lineweaver & Burk (1934). The concentrations of folic acid represent the values obtained with the different organisms after conjugase treatment of the heated extract. The three test organisms react to different forms of folic acid (see Methods section).

	Concn. of folic acid (μM)		
	In heated extract		Required for half-maximum methionine synthesis
Assay organism	Before conjugase treatment	After conjugase treatment	
Lactobacillus casei	29	44	1·5
Streptococcus faecalis R	1·0	90	3·0
Pediococcus cerevisiae	1·1	12	0·4

triglutamate was 8 μM, and was not affected by the presence of cobalamin.

The activity of tetrahydropteroyltriglutamate, like that of extract of heated organisms, was inhibited competitively by tetrahydropteroylglutamate (Fig. 3). The K_i for tetrahydropteroylglutamate was 5·3 μM. Tetrahydropteroyltriglutamate did not inhibit the cofactor activity of the extract of heated *E. coli*.

Similar results were obtained with the tetrahydro derivative prepared either from purified synthetic pteroyltriglutamate or from pteroyltriglutamate isolated from natural sources.

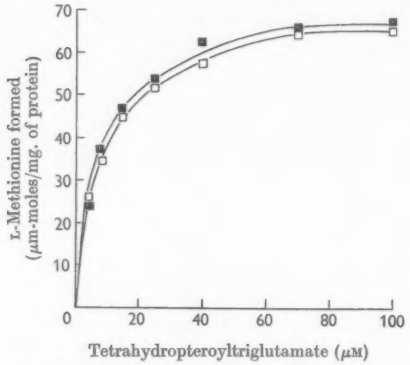

Fig. 2. Tetrahydropteroyltriglutamate as a cofactor of methionine synthesis by ultrasonic extracts of *E. coli* PA15. Incubation time, 2 hr. ■, Without cobalamin; □, with cobalamin (70 $\mu\mu$moles).

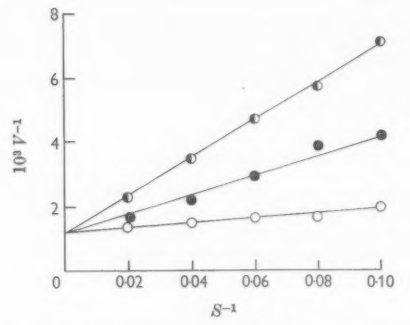

Fig. 3. Effect of tetrahydropteroylglutamate on methionine synthesis by ultrasonic extracts of *E. coli* PA15 with tetrahydropteroyltriglutamate as cofactor. Incubation time, 2 hr. *S*, Tetrahydropteroyltriglutamate concentration (μM); *V*, L-methionine formed (μm-moles/mg. of protein). Concentration of tetrahydropteroylglutamate: ○, nil; ●, 15 μM; ◐, 30 μM.

N^{10}-*Formyltetrahydropteroyltriglutamate*. This folic acid derivative was also active in promoting methionine synthesis in the absence of cobalamin and was unaffected by added cobalamin. The activity of the N^{10}-formyl derivative was approximately the same as that of tetrahydropteroyltriglutamate; the K_m was 8 μM. Its cofactor activity was also inhibited by tetrahydropteroylglutamate, but the nature of the inhibition has not been examined. N^{10}-Formyltetrahydropteroyltriglutamate does not itself inhibit methionine synthesis with extract of heated organisms as cofactor.

When serine was omitted from the reaction mixture and N^{10}-formyltetrahydropteroyltriglutamate increased to 2·5 μmoles, no methionine was formed. This formyl group was evidently incapable of acting as a source of C_1 units for methionine formation.

N^5-*Formyltetrahydropteroyltriglutamate*. This compound also showed cofactor activity which was entirely independent of added cobalamin; the K_m was 1·6 μM (Fig. 4).

Tetrahydropteroylglutamate acted as a competitive inhibitor of N^5-formyltetrahydropteroyltriglutamate in methionine synthesis; the K_i was 1·9 μM. Like the other triglutamate derivatives, N^5-formyltetrahydropteroyltriglutamate did not inhibit the activity of the heated extract of *E. coli*.

The formyl group of this compound, like that of the N^{10}-formyl derivative, was unable to serve as the source of the C_1 unit for methionine synthesis in the absence of serine.

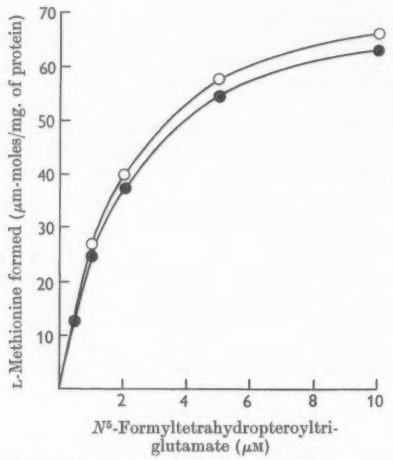

Fig. 4. N^5-Formyltetrahydropteroyltriglutamate as cofactor of methionine synthesis by ultrasonic extracts of *E. coli* PA15. Incubation time, 2 hr. ●, Without cobalamin; ○, with cobalamin (70 $\mu\mu$moles).

Tetrahydropteroylheptaglutamate. The reduced solution of pteroylheptaglutamate, presumed to contain tetrahydropteroylheptaglutamate, showed cofactor activity for methionine synthesis at concentrations of the same order of magnitude as were active with tetrahydropteroyltriglutamate.

Unreduced folates. Pteroylglutamate, pteroyltriglutamate, and N^{10}-formylpteroyltriglutamate showed no cofactor activity in the synthesis of methionine, nor did they affect the activity of the heated extract of organisms.

Effect of cobalamin on the inhibition caused by tetrahydropteroylglutamate. The inhibition of methionine formation caused by tetrahydropteroylglutamate when polyglutamate compounds served as the folic acid cofactors was overcome by cobalamin (Table 2). Tetrahydropteroylglutamate, however, is itself an active cofactor under these conditions and the nature of the action of cobalamin is still unknown.

Stability of the active compounds. The cofactor activity of the extract of heated organisms is stable for several months in air at $-14°$. Incubation for 18 hr. at 30° in air caused a 10–15 % loss of activity, whereas the activity of tetrahydropteroylglutamate was completely destroyed under these conditions (Table 3). Of the reduced triglutamates the N^5- and N^{10}-formyltetrahydropteroyltriglutamates were as stable as the heated extract, but the activity of tetrahydropteroyltriglutamate was considerably reduced and resembled the monoglutamate in this respect (Table 3).

Methionine synthesis by extracts of acetone-dried Escherichia coli *PA* 15

The cofactor activity of some of the above compounds has also been examined qualitatively, though not quantitatively, by using extracts of acetone-dried organisms of *E. coli* PA 15 as source of the enzymes for methionine synthesis. With such extracts tetrahydropteroylglutamate is only active when the organisms have been grown originally in the presence of cobalamin; extract of heated *E. coli* on the other hand supports methionine synthesis whether or not the organisms used in making the acetone-dried powder have been grown with cobalamin. Tetrahydropteroyltriglutamate and its N^5-formyl derivatives were active with extracts of acetone-dried organisms grown in the absence of cobalamin; that is, they resemble the heated extract of *E. coli* rather than tetrahydropteroylglutamate (Table 4).

Table 2. *Effect of cobalamin on the inhibition of the cofactor activity of several conjugated forms of folic acid by tetrahydropteroylglutamate*

Reaction mixtures contained 1 μmole of tetrahydropteroylglutamate, 0·7 μm-mole of cobalamin and 0·1 ml. of the extract of heated *E. coli* when added. Incubation was for 2 hr.

	L-Methionine formed (μm-moles/mg. of protein)		
Source of folic acid cofactor	No additions	Tetrahydropteroylglutamate	Tetrahydropteroylglutamate + cobalamin
None	2	2	58
Extract of heated *E. coli*	68	4	63
Tetrahydropteroyltriglutamate (0·5 mM)	96	4	90
N^{10}-Formyltetrahydropteroyltriglutamate (0·5 mM)	96	3	93
N^5-Formyltetrahydropteroyltriglutamate (0·1 mM)	99	3	94

Table 3. *Stability of folic acid cofactors*

Samples of the folic acid compounds in 0·02 M-phosphate buffer, pH 7 0, and of extracts of heated *E. coli* were incubated at 30° in air for 18 hr. at the stated concentrations. The reaction mixture for testing tetrahydropteroylglutamate both before and after treatment contained 0·7 μm-mole of cobalamin. Incubations were for 2 hr. with ultrasonic extracts of *E. coli* PA15.

	Concn. of folic acid cofactor		L-Methionine formed (μm-moles/mg. of protein)	
Source of folic acid cofactor	During treatment in air (mM)	During test for methionine formation (μM)	Untreated	Treated
Tetrahydropteroylglutamate	5·0	300	40	0
Tetrahydropteroyltriglutamate	0·2	15	51	12
N^{10}-Formyltetrahydropteroyltriglutamate	0·2	15	50	43
N^5-Formyltetrahydropteroyltriglutamate	0·5	2·4	60	53
Extract of heated *E. coli*	0·01–0·09*	0·45–3·4*	58	51

* Estimated microbiologically with three organisms after treatment with conjugase.

Methionine synthesis by ultrasonic extracts of E. coli 121/176

When ultrasonic extracts of methionine-grown *E. coli* 121/176, a cobalamin or methionine auxotroph, were employed, the extract of heated organisms, tetrahydropteroylglutamate and tetrahydropteroyltriglutamate all required the addition of cobalamin to the reaction mixtures before methionine synthesis exceeded about 20% of the maximum (Table 5). Inhibition of the activity of the heated extract and the derivatives of tetrahydropteroyltriglutamate by tetrahydropteroylglutamate was not found with ultrasonic extracts of this strain; cobalamin must be present for optimum synthesis of methionine and cobalamin is known to prevent such inhibition with ultrasonic extracts of strain PA 15. However, the inhibitory properties of tetrahydropteroylglutamate may be inferred from the fact that in the absence of cobalamin the value obtained for methionine formation in strain 121/176 is least with tetrahydropteroylglutamate as the cofactor. In addition the value with extract of heated organisms as the cofactor was decreased by addition of the reduced monoglutamate.

DISCUSSION

Qualitative comparison of the results obtained with the synthetic compounds, tetrahydropteroyltriglutamate, N^{10}-formyltetrahydropteroyltriglutamate and N^5-formyltetrahydropteroyltriglutamate, with those obtained with heated extract of *E. coli* and with tetrahydropteroylglutamate shows that the three triglutamates resemble in behaviour the heated extract rather than tetrahydropteroylglutamate. First, they are quite independent of cobalamin for their cofactor activity in methionine synthesis, except in extracts of strain 121/176 where cobalamin is also required for the heated extract to be active. Secondly, their activity is inhibited competitively by tetrahydropteroylglutamate.

A quantitative comparison of the cofactor activities of the synthetic compounds (Table 6) confirms the similarity of the triglutamates to the

Table 4. *Folic acid cofactors in methionine synthesis by extracts of acetone-dried* Escherichia coli *PA* 15

Incubations were performed as described in the Methods section for 3 hr.

Folic acid compound	L-Methionine formed (μm-moles/mg. of protein) by extracts prepared from acetone-dried organisms grown	
	Without cobalamin	With cobalamin
Tetrahydropteroylglutamate (0·5 mM)	3	25
Tetrahydropteroyltriglutamate (75 μM)	45	33
N^5-Formyltetrahydropteroyltriglutamate (5 μM)	40	—
Extract of heated *E. coli* (0·1 ml.)	36	35

Table 5. *Effect of cobalamin on methionine synthesis by ultrasonic extracts of* Escherichia coli 121/176 *with various folic acid cofactors*

Incubation was for 4 hr. Reaction mixtures contained 70 $\mu\mu$moles of cobalamin where indicated.

Folic acid compound	L-Methionine formed (μm-moles/mg. of protein)	
	Without cobalamin	With cobalamin
Tetrahydropteroylglutamate (500 μM)	0	91
Tetrahydropteroyltriglutamate (150 μM)	23	128
Extract of heated *E. coli* (0·15 ml.)	17	80

Table 6. *Concentrations of folic acid cofactors required for half-maximal methionine synthesis by ultrasonic extracts of* Escherichia coli *PA* 15

Values obtained from results given in Table 1, Figs. 2 and 4, and in the text.

Cofactor	Concn. for half-maximal methionine synthesis (μM)
Tetrahydropteroylglutamate	180
Tetrahydropteroyltriglutamate	8
N^{10}-Formyltetrahydropteroyltriglutamate	8
N^5-Formyltetrahydropteroyltriglutamate	1·6
Extract of heated *E. coli*	0·4–3·0

active components of the extract of heated *E. coli*. During the reduction of the pteridine ring two stereoisomers are formed by the introduction of a new asymmetric centre at C-6. It is possible that only one of these is active as a cofactor and in this event the concentrations given for the synthetic compounds in Table 6 should be halved. Although the total folic acid concentration corresponding to the amount of heated extract of *E. coli* required for half-maximum methionine synthesis depends to some extent upon the assay organism used, it is clearly of the order of 1 μM. There is also the possibility that some of this folic acid may be in forms devoid of cofactor activity and that the true figure for half-maximal activity should be lower. Of the synthetic compounds tested, N^5-formyltetrahydropteroyltriglutamate was active at concentrations similar to those found in the heated extract. The stability of this compound also resembled that of the heated extract and it may therefore be a component of the extract of heated *E. coli*. Tetrahydropteroyltriglutamate on the other hand was considerably less stable than the heated extract. The N^{10}-formyl derivative was only one-fifth as active as the N^5-formylated compound, but it appeared to be as stable as the latter. The N^{10}-formyl derivative of tetrahydropteroylmonoglutamate is particularly unstable (Rabinowitz, 1960); the apparent stability of the triglutamate derivative may be due to the conversion of a small portion into the more active N^5-formyltetrahydropteroyltriglutamate.

In view of the results of Wright (1958) who found that the unreduced pteridine compounds, pteroyltriglutamate and N^{10}-formylpteroyltriglutamate were active in supporting the serine–glycine interconversion in *C. sticklandii*, it is noteworthy that only the reduced compounds are active for methionine synthesis. It seems probable that extracts of *C. sticklandii* were capable of bringing about the reduction enzymically under the conditions used.

The fact that not only tetrahydropteroyltriglutamate but also both its N^5- and N^{10}-formyl derivatives are active as cofactors raises the question of their mechanism of action. For tetrahydropteroylglutamate the suggestion has been made (Kisliuk & Woods, 1960) that $N^{5:10}$-methylenetetrahydropteroylglutamate, which is known to be formed from serine and tetrahydropteroylglutamate (Blakley, 1958), transfers the methylene group to homocysteine, with an accompanying reduction, to form methionine. A similar mechanism might apply to tetrahydropteroyltriglutamate, but the formylated derivatives would have first to be deformylated before this mechanism could operate. If this were the case it is difficult to see why N^5-formyltetrahydropteroyltriglutamate should be active at concentrations one-fifth of those required for tetrahydropteroyltriglutamate. For the N^{10}-formyl compound, which is active at the same concentration as the unsubstituted triglutamate, deformylation or conversion into the N^5-formyl compound may be a necessary prerequisite for cofactor activity.

It appears possible that the N^5-formyl group is concerned with the attachment of the folic acid cofactor to the apoenzyme and that it is the N^{10}-position which is active in the transfer of the C_1 unit. It may be suggested that N^5-formyltetrahydropteroyltriglutamate accepts a C_1 unit from serine at the hydroxymethyl level of oxidation forming N^5-formyl-N^{10}-hydroxymethyltetrahydropteroyltriglutamate and that this latter compound transfers the hydroxymethyl group to homocysteine with an accompanying reduction, to yield methionine.

It is not surprising that neither of the formyl derivatives of tetrahydropteroyltriglutamate is able to act as donor of a C_1 unit in methionine synthesis when it is remembered that in these compounds the C_1 unit is at a formyl, that is formic acid, level of oxidation whereas the C_1 transfer from serine to yield methionine occurs at the hydroxymethyl (formaldehyde) level.

Cobalamin is required for methionine formation by extracts of *E. coli* 121/176 with the reduced triglutamates or the extract of heated organisms as well as with tetrahydropteroylglutamate as the source of cofactor for C_1 transfer. This supports the view that cobalamin is concerned in the reduction on transfer of C_1 units and not in the elaboration of a competent folic acid coenzyme from tetrahydropteroylglutamate.

SUMMARY

1. Tetrahydropteroyltriglutamate, its N^5- and N^{10}-formyl derivatives and an extract of heated *Escherichia coli* are each able to serve as source of folic acid cofactor for methionine synthesis by ultrasonic extracts of *E. coli* PA 15 in the absence of added cobalamin. Tetrahydropteroylglutamate requires added cobalamin for activity.

2. Pteroylglutamate, pteroyltriglutamate, and N^{10}-formylpteroyltriglutamate are devoid of cofactor activity.

3. The K_m values for the synthetic compounds are: tetrahydropteroylglutamate (with 77 $\mu\mu$moles of cobalamin), 180 μM; tetrahydropteroyltriglutamate and its N^{10}-formyl derivative, both 8 μM; N^5-formyltetrahydropteroyltriglutamate, 1·6 μM. The folic acid concentration of a reaction mixture giving half-maximal methionine synthesis with extract of heated *E. coli* is 0·4–3 μM, depending upon the assay organism employed.

4. Tetrahydropteroylglutamate is a competitive inhibitor of the cofactor activity of extract of heated *E. coli*, and of tetrahydropteroyltriglutamate and its N^5-formyl derivative. It also inhibits the activity of the N^{10}-formyl compound. In all cases the inhibition is overcome by the addition of cobalamin.

5. The cofactor activity of tetrahydropteroylglutamate and tetrahydropteroyltriglutamate is unstable to exposure to air at 30° for 18 hr., whereas the activities of extract of heated *E. coli* and of N^{10}- and N^5-formyltetrahydropteroyltriglutamate are relatively stable under these conditions.

6. Cobalamin is required for optimum formation of methionine by ultrasonic extracts of *E. coli* 121/176 (a cobalamin- or methionine-requiring auxotroph) when the derivatives of pteroyltriglutamate, tetrahydropteroylglutamate or an extract of heated organisms supply the necessary folic acid cofactor.

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On the Mechanism of Activation of Phosphoglucomutase by Metal Ions

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Phosphoglucomutase is a widely distributed enzyme which catalyses the interconversion of glucose 1-phosphate and glucose 6-phosphate; it requires a coenzyme, glucose 1:6-diphosphate (Cardini, Paladini, Caputto, Leloir & Trucco, 1949). The enzyme has been purified from rabbit skeletal muscle (Najjar, 1948; Jagannathan & Luck, 1949).

Cori & Cori (1937) first showed that Mg^{2+} ion activates the enzymic conversion of glucose 1-phosphate into glucose 6-phosphate. With the extracted enzyme, both as crude extracts and purified preparations, different authors reported differing effects of Mg^{2+} ion. According to Stickland (1949) and Jagannathan & Luck (1949) no significant activity is present in the absence of Mg^{2+} ion, Mn^{2+} ion or other bivalent metals. Cori, Colowick & Cori (1938) and Najjar (1948), on the other hand, reported that the enzyme is partially active in the absence of added metal, but that activation is obtained by adding Mg^{2+} ion in optimum concentrations (15-fold activation with electrodialysed crude extracts; fourfold activation with the purified enzyme).

The effect of several other metals has also been reported. In crude muscle extracts, Stickland (1949) claimed a further activating effect of a

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second metal (Cr^{3+} or Al^{3+} ions) in addition to Mg^{2+} ion. Recently McCoy & Najjar (1959) reported that in the yeast enzyme Zn^{2+} ion could partially replace Mg^{2+} ion as activator.

Phosphoglucomutase appears to be an extremely complex system. Activation of the enzyme has been obtained with Mg^{2+} ion (or other bivalent metals), 'second metals', chelating agents added with the test reagents (Najjar, 1948; Sutherland, 1949), and further by preincubation of the enzyme with ethylenediaminetetra-acetic acid or histidine and magnesium (Milstein, 1960*b*, 1961; Robinson & Najjar, 1960). Reinvestigation of some of these effects was undertaken to clarify some controversial results and to define their nature more precisely. In this paper some observations on the activation by metals are described.

Magnesium is found to be essential for activity, thus confirming the results of Stickland (1949) and Jagannathan & Luck (1949). Some of the metal complexes formed with the components of the system have been studied, and their implication in the mechanism of the activation is discussed. The activation by a 'second metal' was also found with pure phosphoglucomutase. It is suggested that its mechanism is similar to that of the activation by chelating agents.

MATERIALS AND METHODS

Abbreviations used are: FDNB, 1-fluoro-2:4-dinitrobenzene; DNP, 2:4-dinitrophenyl radical; tris, 2-amino-2-hydroxymethylpropane-1:3-diol; EDTA, ethylenediaminetetra-acetic acid.

Protein was estimated by absorption at 278 mμ, the extinction being taken as 7·70 for a 1% solution (Najjar, 1948).

Glucose 1-phosphate and glucose 6-phosphate (British Drug Houses Ltd.) were freed from metals before use. The solution (about 10 mg./ml.) was passed through an Amberlite IR-120 column, 1 cm. wide and 12 cm. high, which was previously washed with acid and transformed into the Na^+ or K^+ form. The potassium salt was used unless otherwise indicated.

Commercial Roche L-cysteine–HCl was recrystallized from a constant-boiling HCl solution. The pH was adjusted to 7·5 with tris before use.

DL-Histidine–HCl was recrystallized from water and washed with acetone and ether.

Enzyme preparations. Phosphoglucomutase was prepared from rabbit skeletal muscle according to Najjar (1948). In general the method was found to reproduce the original results of Najjar, but the amount of crystalline material obtained was usually very low; however, the specific activity after the second crystallization was 26–27 units/mg. of protein, 26 being recorded by Najjar. The preparations proved to be homogeneous in the ultracentrifuge under a variety of conditions. Before use the enzyme solution was dialysed against glass-distilled water.

In all calculations a molecular-weight value for phosphoglucomutase of 74 000 (Lowry, Keller & Taylor, 1956) was used.

'Metal-free' phosphoglucomutase was prepared by the following procedure. The purified enzyme was dialysed against large volumes of glass-distilled water and then against 1 mM-tris–HCl, pH 7·5. It was then passed through an Amberlite IRC-50 column in the tris form, equilibrated with 1 mM-tris–HCl, pH 7·5. The metal content of the preparation after this treatment was negligible according to the 8-hydroxyquinoline test (Milstein, 1960*a*). The specific activity of the enzyme did not change with this treatment.

Measurements of enzyme activity. Unless otherwise stated, the incubation mixture contained: glucose 1-phosphate (containing as an impurity the coenzyme glucose 1:6-diphosphate in sufficient amounts), at a concentration of 6·5 or 0·5 mM depending on the method used for acid-labile phosphate determination; 1·5 mM-$MgSO_4$; 25·0 mM-L-cysteine–HCl or DL-histidine–HCl adjusted to pH 7·5 with tris immediately before use; enzyme as indicated in each case. The final volume was 0·4 ml.

The reaction was started by adding the enzyme to the incubation mixture. After incubation for 5 min. at $30 \pm 0{\cdot}1°$ acid-labile phosphate was determined according to Najjar (1948).

When low concentrations of glucose 1-phosphate were used, a modification of the Fiske & Subbarow (1925) method was used for phosphate determinations (Bartlett, 1959). The modification involves colour development, after all the reagents have been added, in a boiling-water bath for 7 min. The sensitivity with this simple modification increases tenfold. Since the activity determinations assume stability of glucose 6-phosphate, its stability was tested under the conditions used for colour development. Glucose 6-phosphate has been reported to be very stable to acid hydrolysis (Robison, 1932). Even in the presence of 4·5N-H_2SO_4 at 100° the 6-ester hydrolyses very slowly (Table 1, Expt. A). Under Bartlett's conditions of colour development, i.e. in the presence of molybdate and reducing agent in acid at 100° for 7 min., the stability of the

Table 1. *Stability of glucose 6-phosphate*

Glucose 6-phosphate containing 35·8 μg. (Expt. A) or 17·9 μg. (Expt. B) of total phosphorus, with the additions stated, in a final volume of 1·1 ml. (Expt. A) or 5 ml. (Expt. B), was incubated in a boiling-water bath. Inorganic phosphate was determined in the hydrolysate according to Fiske & Subbarow (1925) (Expts. A, B_1) or according to Bartlett (1959) (Expt. B_2).

Expt.	Additions	Time of incubation (hr.)	Inorganic phosphate (μg. of P)	Hydrolysis (%)
A	Blank	0	1·9	0
	4·5N-H_2SO_4	1	3·6	4·7
	4·5N-H_2SO_4	2	5·0	8·6
	4·5N-H_2SO_4	3	7·6	15·9
	4·5N-H_2SO_4	5	9·8	22·0
		(min.)		
B_1	Blank	0	2·0	0
B_2	1N-H_2SO_4 + 0·25% $(NH_4)_2MoO_4$ + 0·2 ml. of reducing agent	7	2·1	0·56

ester is also satisfactory (Table 1, Expt. B). As a final check of the applicability of the method, duplicate assays of enzymic activity were made and phosphate was estimated according to Bartlett, colour development being carried out for 7 and 17 min. at 100°. The values differed by less than 3% (3·38 and 3·30 μg. respectively).

The unit of enzymic activity used throughout was that used by Najjar, i.e. the amount of enzyme which under standard conditions of assay produces a decrease of 32 μg. of acid-labile phosphorus in 5 min.

Equilibrium dialysis experiments. The binding capacity of phosphoglucomutase for Mg^{2+} ion was measured by equilibrium dialysis. The experiments were carried out in special cells (for which I am indebted to Dr M. Dixon). These consisted of two circular pieces of Perspex in which a cylindrical depression of 2·8 cm. diameter was cut. The two pieces could be held together tightly by three screws. In use, a cellophan membrane soaked with 8-hydroxyquinoline and glass-distilled water was fixed between both pieces, dividing the space into two compartments of 2·25 ml. each. Two small holes through the sides of the compartments allowed the introduction and removal of samples. The actual experiment consisted in equilibrating (at 4°) the enzyme, placed in one compartment, with a solution in the other compartment. Equilibration was complete after 3 days (occasionally the cells were gently shaken). Samples from the compartment not containing protein were taken and assayed for Mg^{2+} ion. A blank was run without protein, and was used to evaluate the binding capacity of the cellophan membrane, which was very small. Binding was expressed as ν, the number of Mg^{2+} ions bound to one molecule of phosphoglucomutase, as calculated from the total amount of metal minus the free metal, corrected for the blank.

Dissociation constants of glucose 1-*phosphate–magnesium and glucose* 6-*phosphate–magnesium complexes.* The estimation of the dissociation constant of these magnesium complexes was made by the method of Burton (1959) with some modifications. The maximum of the 8-hydroxyquinoline–Mg chelate at 253 mμ (Milstein, 1960*a*) was used instead of that at 360 mμ. Mg^{2+} ion was added to a sample containing 8-hydroxyquinoline and the glucose phosphate, and increase in the extinction at 253 mμ was plotted against the amounts of Mg^{2+} ion added.

The dissociation constant is calculated (Burton, 1959) from the slopes of the resulting curves at the origin

$$K = \frac{A - B}{[\mathrm{G} - \mathrm{P}] \times B} \qquad (1)$$

in which A is the initial slope when no glucose phosphate is present, B the initial slope in the presence of glucose phosphate and $[\mathrm{G} - \mathrm{P}]$ is the total glucose phosphate concentration.

Reaction of 1-*fluoro*-2:4-*dinitrobenzene with phosphoglucomutase.* The effect of FDNB on phosphoglucomutase was studied in the presence of EDTA to avoid any protective effect of metal impurities, since extremely stable metal complexes have been shown to be formed with the enzyme (Milstein, 1960*b*, 1961) and FDNB reacts with free groups. Before use, phosphoglucomutase was preactivated (Milstein, 1960*b*) by incubation with 0·7 mM-EDTA, pH 7·5, for 1 hr. at 30°.

Enzyme (300 μg.) was incubated in the presence of 0·3 mM-EDTA with 5·4 mM-FDNB at 30°. The final volume was 0·94 ml. The addition of FDNB was made as a small volume of a concentrated ethanolic solution giving a final ethanol concentration of 4·5%. The stability of the enzyme in the absence of FDNB was tested and no fall in activity was observed. The effect of 1·9 mM–$MgSO_4$ on the reaction with FDNB was also studied.

The effect of FDNB was followed by measuring the residual enzyme activity at different times after the addition of the reagent. Samples (2·1 μg.) of enzyme were taken and assayed for activity in the presence of cysteine as activator.

Since the concentration of FDNB is much higher than the concentration of the enzyme, the velocity of the reaction should be approximately proportional to the concentration of phosphoglucomutase. If the reaction follows apparently first-order kinetics, its rate is a linear function of the active enzyme concentration

$$\frac{-\mathrm{d}[E]}{\mathrm{d}t} = k'[E]$$

E being the concentration of active enzyme. In the integrated form the equation becomes

$$\log \frac{[E]}{[E_0]} = -kt \qquad (2)$$

where $[E]/[E_0]$ is the amount of active enzyme relative to the total at any chosen time and is thus equal to A/A_0, A and A_0 being the enzyme activities at time t and initial time of the reaction respectively. The graph of the logarithm of the residual activity plotted as a function of time gives a straight line with a slope of $-k$. The comparison of the values of k in the presence and the absence of Mg^{2+} ion gives a quantitative estimate of the protective effect of the metal towards the inhibition by FDNB.

The partially inactive DNP–enzyme used in the experiment in Fig. 5 was prepared as follows: 9·5 mg. of phosphoglucomutase was incubated at 23° in the presence of 1 mM-EDTA at pH 5·8. At time 0, 5 ml. of saturated aqueous FDNB and 1 drop of liquid FDNB were added to make the final volume 6·1 ml. The solution was stirred very gently (8 turns/min.) for 5·5 hr. During the reaction a yellow precipitate was slowly formed. At 1 and 5·5 hr. samples of the supernatant were taken and used for determinations of α-amino nitrogen and activity. The precipitate was collected at the end of the reaction, and its α-amino nitrogen was also determined by the ninhydrin method (Chibnall, Mangan & Rees, 1958). For these determinations the samples were extracted with ether and hydrolysed (15 hr. with 6N-HCl in sealed tubes, at 105°). The relative amounts of protein in the fractions were calculated from the α-amino nitrogen and compared with the activity.

After reaction for 1 hr., 85% of the protein remained in solution, containing 69% of the original activity. After 5·5 hr., 24% of the enzyme remained in solution, containing 7·6% of the original activity. The precipitate was inactive. The fall in the activity is then slightly quicker than the formation of the insoluble material. This insoluble material, after hydrolysis, was extracted with ether and treated as for the determinations of N-terminal groups in proteins by the FDNB method (Fraenkel-Conrat, Harris & Levy, 1955). There was no DNP-amino acid in the ether. The aqueous layer containing the DNP-amino acids (except the N-terminal) was subjected to chromatography in butanol–acetic acid–water (4:1:5) with markers of DNP-lysine and

Table 2. *Requirement for a metal in the phosphoglucomutase reaction*

Enzyme activity was determined by the standard assay, except for the addition of Mg^{2+} ion and EDTA (indicated below). Cysteine was used as activator.

Expt.	Enzyme (μg.)	Additions	Activity (μg. of P/5 min.)	Relative activity (% of activity with optimum $[Mg^{2+}]$)
A	*	None	−2·4	−3·5
A	*	$MgSO_4$ (1·5 mM)	65·8	100·0
B	5·1	None	2·7	4·3
B	5·1	$MgCl_2$ (0·37 mM)	57·6	92·0
C	2·7	None	10·2	7·8
C	2·7	$MgCl_2$ (1·5 mM)	> 75·0	—
C	1·1	$MgCl_2$ (1·5 mM)	65·5	100·0
C	2·7	EDTA (1·0 mM)	1·0	0·8
D	2·1	None	5·4	10·1
D	2·1	$MgSO_4$ (1·5 mM)	53·5	100·0
D†	10·1	$MgSO_4$ (1·5 mM) + EDTA (0·05 mM)	37·6	100·0
D†	10·1	$MgSO_4$ (1·5 mM) + EDTA (0·15 mM)	37·1	98·5
D†	10·1	$MgSO_4$ (1·5 mM) + EDTA (0·50 mM)	34·6	92·0
D†	10·1	$MgSO_4$ (1·5 mM) + EDTA (4·0 mM)	0·4	1·6

* Amount not determined. † No cysteine or histidine present.

histidine. The two DNP-amino acids DNP-lysine and DNP-tyrosine were determined by their absorption in the ultraviolet, and free histidine was determined according to Fraenkel-Conrat & Singer (1956). No free histidine was found, implying complete reaction of this amino acid with FDNB; 6 molecules of DNP-lysine and about 5 of DNP-tyrosine per molecule of original enzyme were found, although with a high degree of error in the determination of the latter, due to its much lower absorption coefficient.

Under these conditions then, the reaction is not specific for any one of the groups involved, and there is the possibility of reaction with more than one essential group, or with groups involved in the tertiary structure.

Portions of the supernatant of the above experiment were taken after 1 min., 1 hr. and 5·5 hr. and used for determinations of $K_{app.}$ (see Results section) for magnesium shown in Fig. 5.

RESULTS

Activation by magnesium

When no Mg^{2+} ion was added to the assay mixture, the activity was in general very low, ranging from 0 to 3% of its optimum in the presence of Mg^{2+} ion, values which are well within the error of the activity determinations. The result, in agreement with that of Stickland (1949), who used a crude preparation, and with that of Jagannathan & Luck (1949) using their own preparation of purified enzyme, contrasts with that of Najjar (1948), who, using a preparation similar to that used in this work, reported only a fourfold activation by Mg^{2+} ion.

In some instances, however, values as high as 10% of the optimum activity were obtained in the absence of magnesium. Neither purification of the enzyme ('metal-free' enzyme) nor purification of the substrate was effective in reducing the activity in the absence of magnesium to zero.

Since the important point was to show the absolute requirement of a metal for activity, the effect of EDTA was studied in these cases, to see whether the activity was due to residual traces of metals. EDTA is a well-known complexing agent of metals, including the bivalent metals. It is an activator of phosphoglucomutase when used in low concentrations (Milstein, 1961), but when the concentration of EDTA is increased to a value higher than the concentration of Mg^{2+} ion the activity drops to almost zero, owing to chelation and effective removal of the metal. This was found in all cases, including those where absence of Mg^{2+} ion from the assay mixture was not enough to reduce the activity to zero. Furthermore, the small activity observed in the absence of added metals in these few cases can be completely abolished by the addition of 1 mM-EDTA to the assay mixture. Table 2 summarizes these observations, which indicate that the activity in these cases is due to residual traces of metals. Assuming that Mg^{2+} ion is essential for the activity at low concentrations the reaction in the absence of EDTA should be expressible in the reciprocal form as

$$\frac{1}{v} = \frac{1}{V} + \frac{K_{app.}}{V} \cdot \frac{1}{a} \qquad (3)$$

in which V is a constant dependent on the conditions of the reaction, $K_{app.}$ is the apparent Michaelis constant for magnesium, a constant whose significance depends on the mechanism of the activation, a the concentration of the activating metal and v the velocity. A plot of $1/v$ against $1/[Mg^{2+}]$ yields a straight line from which $K_{app.}$ can be obtained (Fig. 1). This shows again that the activation should be considered as an essential activation (Segal, Kachmar & Boyer, 1952), and

phosphoglucomutase should be included among the 'true metal–enzymes' as described by Malmström & Rosenberg (1959).

The value of $K_{app.}$ (50 μM) is some 1/200 of the dissociation constants of the magnesium complexes of the substrates glucose 1-phosphate and glucose 6-phosphate. These last values were determined by the method of Burton (1959), but with the use of the absorption peak of the 8-hydroxyquinoline chelate at 253 mμ because of its higher sensitivity (Fig. 2). The values calculated from equation (1), as averages of duplicate determinations, are:

$$K^{Mg}_{\text{for glucose 6-phosphate}} = 16 \text{ mM};$$
$$K^{Mg}_{\text{for glucose 6-phosphate}} = 11 \text{ mM}.$$

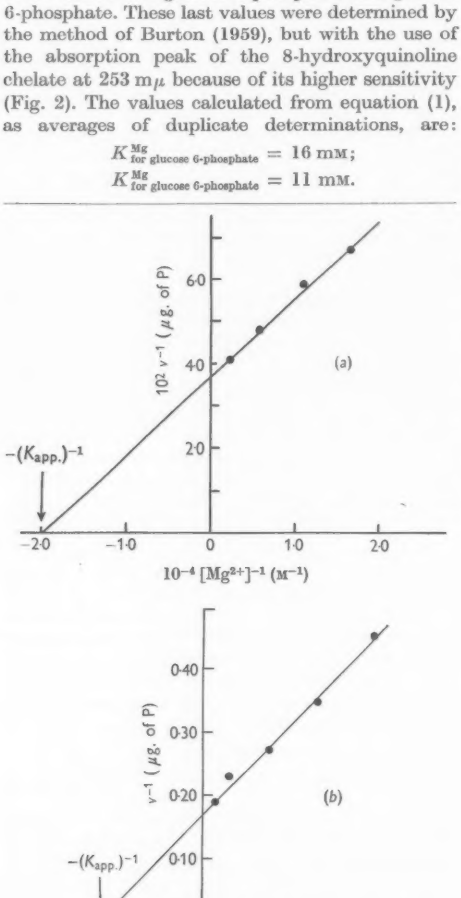


Fig. 1. Effect of Mg^{2+} ion concentration on the reaction rate (reciprocal plots). The enzyme was incubated for 5 min. at 30° in the presence of 25 mM-histidine and Mg^{2+} ion as indicated at pH 7·5. Velocity is expressed as the amount of acid-labile phosphate disappearing. (*a*) 3·7 μg. of phosphoglucomutase and 5·5 mM-glucose 1-phosphate; (*b*) 0·7 μg. of phosphoglucomutase and 0·5 mM-glucose 1-phosphate. Phosphate determinations were according to Bartlett (1959).

The similarity between the two constants suggests that Mg^{2+} ion should not significantly affect the overall equilibrium of the enzyme reaction.

That phosphoglucomutase binds Mg^{2+} ion was shown by equilibrium-dialysis methods. The enzyme, in the presence of about an equimolar concentration of Mg^{2+} ion, was dialysed for several days until equilibrium was established; determinations of Mg^{2+} ion in the diffusate gave the concentration of free metal in the enzyme solution (the Donnan effect being insignificant at this concentration of buffer) and, from the total initial Mg^{2+} ion, the amount of bound metal can be calculated by difference. In Fig. 3 is shown the amount of bound metal at various concentrations of free Mg^{2+} ion. It can be seen from the experiment that a phosphoglucomutase–magnesium complex exists, even in the absence of substrate. Its dissociation constant calculated from these data is 0·5 mM. This is only a tentative value, and errors may be expected, mainly due to the fact that Mg^{2+} ion binding is calculated from the differences between two relatively high values, each subject to individual error.

An independent indication that Mg^{2+} ion forms a complex with the protein was obtained from inhibition experiments. Phosphoglucomutase is inhibited by FDNB, and the kinetics of the reaction are shown in Fig. 4. In the presence of an adequate concentration of Mg^{2+} ion for maximum activity

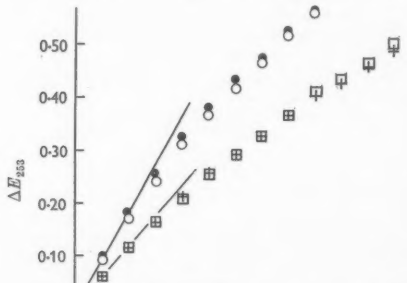


Fig. 2. Determination of the dissociation constants of glucose 1-phosphate and glucose 6-phosphate complexes with Mg^{2+} ion. To a cuvette containing 33 mM-tris–HCl buffer, pH 7·5, 83 μM-8-hydroxyquinoline and the additions indicated below (final volume, 3·0 ml.) $MgCl_2$ was added in successive portions of 20 μl. ●, None; ○, 0·05 M-tris–HCl, pH 7·5; +, 7·3 mM-glucose 6-phosphate (sodium salt), pH 7·5; □, 8·4 mM-glucose 1-phosphate (sodium salt), pH 7·5. Readings were made at 253 mμ after the addition of each portion of $MgCl_2$, against a blank containing the same reagents with $MgCl_2$ omitted.

(1·2 mM-free Mg^{2+} ion, after allowing for the EDTA-binding) the reaction proceeds at a clearly different rate (Fig. 4), indicating that Mg^{2+} ion diminishes the reactivity of the protein towards FDNB, probably through the formation of a phosphoglucomutase–magnesium complex. The value of k (equation 2) is $7{\cdot}1 \times 10^{-4}$ min.$^{-1}$ in the presence of Mg^{2+} ion and $1{\cdot}2 \times 10^{-2}$ min.$^{-1}$ in its absence. This indicates that Mg^{2+} ion produces a 17-fold decrease in the rate constant of the inactivation by FDNB.

If the reaction with FDNB affects groups in the enzyme surface which influence the formation of the enzyme–magnesium complex, a change in the value of the apparent Michaelis constant for magnesium ($K_{app.}$) would be expected. On the other hand, if the inactive DNP-enzyme does not form an enzyme–magnesium complex, or if inhibition does not influence the activation by Mg^{2+} ion, a change in V will occur. In fact only V is affected by the inhibition and the same value of $K_{app.}$ is observed for different degrees of FDNB-inactivated enzyme (Fig. 5). The decrease in V (shown in the legend of Fig. 5) shows that the soluble enzyme is partially inactive before precipitating, since the values

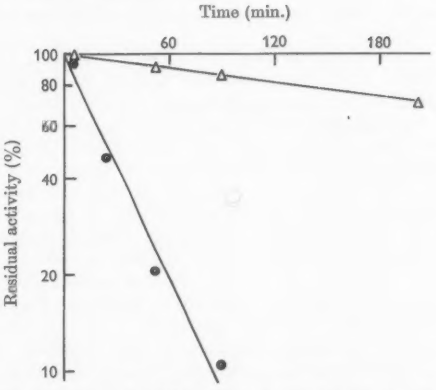


Fig. 4. Rate of inhibition of phosphoglucomutase by FDNB, in the absence and in the presence of Mg^{2+} ions. Phosphoglucomutase (300 μg.), preactivated (1 hr. in 0·7 mM-EDTA at 30°), in 0·3 mM-EDTA. Final volume, 0·94 ml. (containing 4·5% of ethanol). ●, 5·4 mM-FDNB; △, 5·4 mM-FDNB + 1·9 mM-$MgSO_4$. Portions containing 2·1 μg. of enzyme were taken at the indicated times and assayed for activity (activator, cysteine; incubation time for the assay, 4 min.). The original activity of the preparation was 58·5 μg. of P/4 min. Relative activity is expressed as percentage of the activity in the absence of inhibitor.

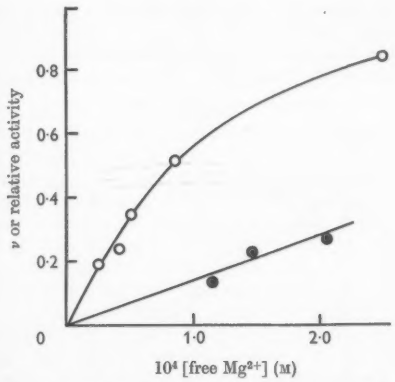


Fig. 3. Comparison of Mg^{2+} ion activation and Mg^{2+} ion binding. ●, Binding of Mg^{2+} ion. 0·17 μmole of 'metal-free' phosphoglucomutase in the presence of 0·01 M-tris–HCl, pH 7·5, and various amounts of $MgCl_2$. Equilibrium dialysis was carried out for 3 days, as described in the Materials and Methods section, and Mg^{2+} ion was determined in the diffusate. A blank without enzyme was made to correct for the binding capacity of the membranes. ○, Activation by Mg^{2+} ion. Phosphoglucomutase (2·5 μg.) was assayed for activity as indicated in the Materials and Methods section. The sodium salt of glucose 1-phosphate was used and each point was determined from a time curve of the disappearance of acid-labile phosphate. Cysteine was used as activator, and Mg^{2+} ion concentrations are corrected for cysteine-binding.

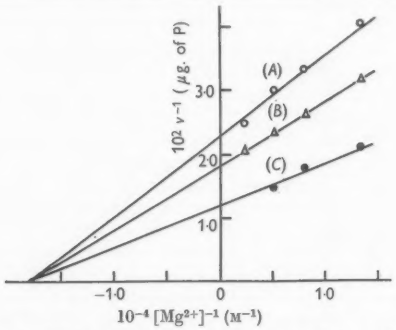


Fig. 5. Effect of FDNB inhibition on $K_{app.}$ and V. Partially inactive DNP-enzyme prepared as described in the Materials and Methods section was diluted 20-fold and samples (0·05 ml.) were assayed for activity with histidine as activator. DNP-phosphoglucomutase obtained after treatment of the enzyme with FDNB, *A*, for 5½ hr., *B*, for 1 hr., *C*, for 1 min. (For measurement of the activity the assay mixture was incubated for 24 min. for *A*, and 2·5 min. for *B* and *C*. Activity is expressed as μg. of phosphate converted in these times.) Values of V (expressed as μg. of phosphate converted in 5 min.) were:

	P (μg./5 min./μg. of original protein)	P (μg./5 min./μg. of soluble protein)
(*A*)	2·6	12·1
(*B*)	32·0	43·5
(*C*)	47·7	47·7

shown have been calculated per milligram of soluble enzyme.

Phosphoglucomutase contains 3 histidine residues, 34–35 lysine residues and 31–32 tyrosine residues per molecule (Anderson & Jollès, 1957).

FDNB readily reacts with histidine, and all the histidine residues are destroyed during FDNB reaction, together with at least 6 lysine and 5 tyrosine residues per molecule of enzyme. Since histidine occurs in the active site of phosphoglucomutase (Milstein & Sanger, 1960) it was of interest to see whether Mg^{2+} ion had any effect on inactivation by photo-oxidation of phosphoglucomutase. It has been suggested by Koshland, Ray & Erwin (1958) that the destruction of one histidine residue by photo-oxidation in the presence of methylene blue is responsible for the inactivation of phosphoglucomutase. In Fig. 6 it is shown that the rate constant of inhibition by photo-oxidation without Mg^{2+} ion is $1{\cdot}7 \times 10^{-2}$ min.$^{-1}$ and with 2 mM free Mg^{2+} ion it is $1{\cdot}2 \times 10^{-2}$ min.$^{-1}$. The effect of Mg^{2+} ion on the photo-oxidation certainly cannot be compared with the protection given by the ion against inhibition by FDNB. The experiment suggests that Mg^{2+} ion does not affect significantly the sensitivity of the histidine presumably involved in the inactivation.

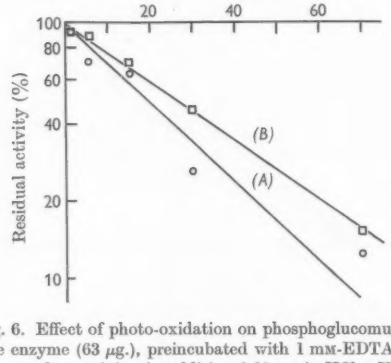


Fig. 6. Effect of photo-oxidation on phosphoglucomutase. The enzyme (63 μg.), preincubated with 1 mM-EDTA, for 1 hr., and containing in addition 0·01 M-tris–HCl, pH 7·5, and 0·8 μg. of methylene blue and the additions mentioned below, was placed under a 150 W tungsten-filament lamp at 30 cm. distance in a dark room. Portions (0·02 ml.) were removed at different times, diluted with 0·4 ml. of iced 0·1 M-histidine, pH 7·5, and the activity of 0·1 ml. of the dilution was immediately assayed, with histidine as activator. Activity is expressed as percentage of the activity of a blank containing in addition 0·02 ml. of tris-HCl, pH 7·5, and kept in the dark during the procedure. (*A*) Contains also 0·02 ml. of 0·1 M-tris–HCl; (*B*) contains also 0·02 ml. of 0·05 M-$MgCl_2$. 100% activity is equal to 33·0 μg. of P/5 min.

Activation by a 'second metal'

Stickland (1949) reported that a second metal (Cr^{3+}, Al^{3+} ions etc.), in addition to Mg^{2+} ion, was essential for the full activity of phosphoglucomutase. The enzyme he used was not the purified preparation and the activation by cysteine was still unpublished when he submitted his paper. The possible relationship of these two apparently independent activations remained obscure.

In the first attempts with the purified phosphoglucomutase I failed to find any activation by either Cr^{3+} or Al^{3+} ion. However, when higher concentrations of enzyme were used the effect became apparent (thanks are due to Dr L. H. Stickland for his advice on this point). With these concentrations of enzyme, however, the velocity is much too high to measure when cysteine is added. Lower enzyme concentrations were therefore used when cysteine was present. Therefore the concentration of enzyme used was adjusted so as to give roughly the same velocity in all cases and the amount of enzyme required then gives an indication of the activity. Thus when only glucose 1-phosphate, Mg^{2+} ion and enzyme were present in the incubation mixture, 96 μg. of the pure phosphoglucomutase was necessary to convert 40 μg. of acid-labile phosphate in 5 min. When the incubation mixture included also 0·25 mM-$CrK(SO_4)_2$ only 9·3 μg. of the enzyme was required for conversion of the same amount of substrate in the same time. Cysteine is much more

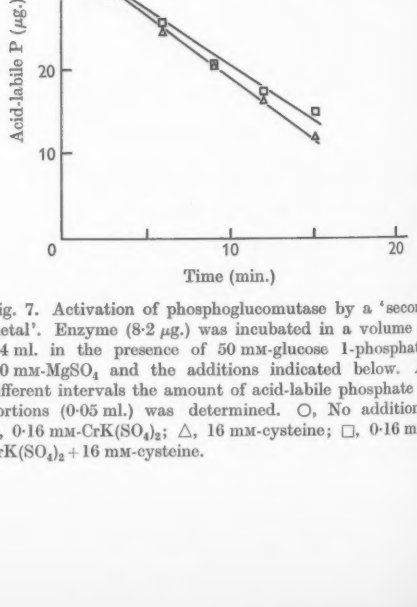


Fig. 7. Activation of phosphoglucomutase by a 'second metal'. Enzyme (8·2 μg.) was incubated in a volume of 0·4 ml. in the presence of 50 mM-glucose 1-phosphate, 1·0 mM-$MgSO_4$ and the additions indicated below. At different intervals the amount of acid-labile phosphate in portions (0·05 ml.) was determined. ○, No additions; ●, 0·16 mM-$CrK(SO_4)_2$; △, 16 mM-cysteine; □, 0·16 mM-$CrK(SO_4)_2$ + 16 mM-cysteine.

Table 3. *Effect of chromium potassium sulphate on the activation of phosphoglucomutase*

The enzyme was incubated with 25 mM-glucose 1-phosphate, pH 7·5, for the indicated period at 30°, in the presence of 1·5 mM-$MgSO_4$ and the additions stated. The amount of acid-labile phosphate was determined and the activity expressed as indicated in the Materials and Methods section.

Expt.	Enzyme (μg.)	$CrK(SO_4)_2$ (0·25 mM)	Tris–HCl (pH 7·5)	Incubation period (min.)	Activity (μg. of P)
A	18	—	—	7·5	3·0
A	18	Fresh	—	7·5	10·0
A	18	4 months old	—	7·5	12·2
B	—	Fresh	—	10·0	0·0
B	18·5	—	—	10·0	4·0
B	18·5	Fresh	—	10·0	9·4
B	18·5	4 months old	—	10·0	14·2
C	17·0	—	—	10·0	2·5
C	17·0	Fresh	—	10·0	11·5
C	17·0	—	10 mM	10·0	5·2
C	17·0	Fresh	10 mM	10·0	10·0

Table 4. *8-Hydroxyquinoline test of chromium potassium sulphate solutions*

The 8-hydroxyquinoline test was as described by Milstein (1960*a*) except that tris–HCl was used as indicated. Final concentration of $CrK(SO_4)_2$ was 54 μM.

Age of the chromic solution	Tris–HCl	ΔE_{253}
Fresh	—	0·265
Fresh	3·5 mM	−0·002
4 months	—	0·272
4 months	3·5 mM	0·000

effective, and when a concentration of 25·0 mM is present instead of the Cr^{3+} ion the amount of enzyme necessary to give the same velocity was only 0·8 μg. If the activity in the presence of cysteine is taken as 100%, the activity in the presence of Cr^{3+} ion is 8·5% and in the absence of either (i.e. with Mg^{2+} ion only) is 0·8%. Higher concentrations of Cr^{3+} ion are inhibitory.

Activation by Cr^{3+} ion and cysteine are not additive (Fig. 7). When cysteine is present, Cr^{3+} ion does not activate, but has a weak inhibitory effect. This suggests that although these two activators are of a very different nature, they are acting in a rather similar way.

Similar observations have been made in some other systems (Altman & Crook, 1953; Snell, 1957; Wada *et al.* 1957). It has been suggested that the tervalent metals are acting as trapping agents of inhibitory metals through their hydrates (Altman & Crook, 1953), or that the 'second metal' competes with the inhibitory metal on the enzyme surface, but the complex thus formed is active (Wada *et al.* 1957).

Solutions of chromium salts are known to undergo changes in hydration on standing. When an aged solution is tested for its activating effect, it can be seen that it is higher than the activating effect of a fresh solution (Table 3, Expts. A and B).

When the 8-hydroxyquinoline test was applied to a solution of a chromic salt, no change in extinction at 253 mμ was observed. However, if the test is made at the same pH but in the absence of tris–HCl, the characteristic peak of the 8-hydroxyquinoline chelates becomes evident (Table 4). The reason for the effect of tris is not clear, but the observation suggests that in the presence of the buffer there are no free Cr^{3+} ions available. However, the activating effect of chromium is not suppressed in the presence of tris–HCl buffer, pH 7·5, in even higher concentrations than those producing the quenching of the 8-hydroxyquinoline chelate peak (Table 3, Expt. C). This would again indicate that the activation is produced not by the Cr^{3+} ions but by the chelating properties of some of its derivatives. However, it should be emphasized that the experiment is by no means conclusive, and the fact that tris itself seems to have some effect introduces a new complication.

It has been shown (Milstein, 1960*b*, 1961) that activation by chelating agents is due to the removal of inhibitory metals; the above-mentioned observations seem to indicate that the explanation offered by Altman & Crook (1953) for a different system (namely that the hydrates of the activating tervalent metals are in fact acting in the same way as the chelating agents) might be the correct one.

DISCUSSION

The results obtained with the equilibrium-dialysis experiments and the protective effect of Mg^{2+} ions during inactivation by FDNB indicate that Mg^{2+} ion forms a complex with the protein. Whether formation of such a complex is responsible for activation of the enzyme remains to be proved.

In fact about 80% of the total activity can be obtained at a concentration of Mg^{2+} ion at which only 30% of the enzyme is present as the magnesium complex, assuming that 1 atom of magnesium combines with 1 molecule of enzyme (see Fig. 3). This is a simple reflexion of a difference between the dissociation constant and the apparent Michaelis constant for Mg^{2+} ion ($K_{app.}$). At first sight this would indicate that the formation of the complex is not responsible for the activation. However, the Michaelis constant is a true dissociation constant only in some cases, depending on the mechanism of the activation. Several authors (Segal *et al.* 1952; Alberty, 1956; Dixon & Webb, 1958) have discussed the kinetic models of activation by metal ions and shown that in many cases the apparent Michaelis constant for the metal is a kinetic expression depending on the enzyme–metal dissociation constant and other parameters. For instance, the kinetic scheme

$$E+M \rightleftharpoons EM; \quad EM+S \rightleftharpoons EMS \rightarrow EM+P \quad (4)$$

corresponds to case II discussed by Dixon & Webb (1958, page 458) for the extreme case in which an ES complex is not formed. The derivation of the value of the apparent Michaelis constant for the metal is

$$K_{app.} = \frac{K_A}{1+(s/K'_s)}. \quad (5)$$

where K_A is the true dissociation constant of the enzyme–metal complex and K'_s the dissociation constant of the enzyme–metal–substrate complex with respect to substrate. This possibility should be verifiable by determining the value of $K_{app.}$ at low substrate concentration. Under these conditions $K_{app.}$ would be expected to rise as the concentration of substrate diminishes, to reach the value of K_A at the limit (substrate concentration nil). An experiment to test this was made. The apparent Michaelis constant for magnesium was determined with the lowest concentration that can be assayed (0·5 mM). The value obtained for the constant is much the same as that obtained under standard conditions with a substrate concentration 10 times as high (Fig. 1). Since the value of the apparent Michaelis constant for substrate under the experimental conditions used has been reported to be of the order of 0·1 mM or lower (Najjar & McCoy, 1958), it would have been expected that, at the concentration of substrate used, the value of $K_{app.}$ would have risen several times, and the difference should have been easily detected.

The fact that the enzyme–metal complex has a dissociation constant lower in value than that of the apparent Michaelis constant for the metal suggested to Malmström, Vängård & Larsson (1958) that the above-mentioned mechanism was operative in the metal activation of enolase, despite the fact that Would & Ballou (1957), postulating a similar mechanism, found that K_s did not change with magnesium concentration.

With phosphoglucomutase, the results indicate a rather more complex mechanism. Thus if, in addition to equation (4), the following steps are also present

$$E+S \rightleftharpoons ES; \; ES+M \rightleftharpoons ESM \quad (6)$$

with a value of K'_A for the dissociation constant of ESM, different from K_A, the velocity of the reaction (given by Dixon & Webb, 1958, page 466) will be

$$v = \frac{ke}{1+K_sK_A/as[1+(a/K_A)+(s/K_s)]}. \quad (7)$$

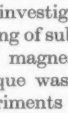
The value of $K_{app.}$ derived from this equation is

$$K_{app.} = \frac{K_A[1+(K_s/s)]}{(K_A/K'_A)+(K_s/s)}. \quad (8)$$

For such a mechanism the value of $K_{app.}$ will depend on both K_A and K'_A, and also on K_s and s. However, when the concentration of the substrate is high in relation to K_s, and $K_A > K'_A$, the value of $K_{app.}$ becomes independent of the substrate concentration, and at the limit (infinite substrate concentration) $K_{app.}$ is the same as K'_A.

Returning to the conditions of measurement of $K_{app.}$, from equation (8) at the lowest possible concentrations of glucose 1-phosphate (0·5 mM, see Fig. 1), even assuming this is three times the value of K_s, the value of $K_{app.}$ could differ by less than 30% from the value at ten times this substrate concentration (5 mM, see Fig. 1). Such a difference is within the experimental error. The simple mechanism, including the steps shown in equations (4) and (6), explains the experimental results satisfactorily, but more complex models are not excluded.

A decrease in the dissociation constant for magnesium on passing from the binary to the ternary complex (EMg to EMgS) means that the affinity for the metal has been increased owing to the presence of the substrate. This might be because the phosphate group of the substrate is being used as a new bonding group of magnesium



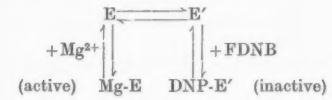
or because of a change in the enzyme configuration during substrate binding [this type of change has been discussed by Koshland (1959)].

A possible way to investigate this point further would be by the binding of substrate in the presence and the absence of magnesium by equilibrium dialysis. This technique was used by Malmström (1955) in model experiments with albumin, and on

enolase. Although the model experiment gave a positive answer, his experiments with enolase showed no effect of the presence of magnesium on substrate binding. In our experiments the amount of substrate bound to the enzyme was measured by the total phosphorus determined in the diffusate, and also by radioactive measurements with [^{14}C]-glucose phosphate. The enzyme in high concentrations was allowed to equilibrate in an equilibrium-dialysis cell with an equimolar concentration of substrate, in the presence and in the absence of Mg^{2+} ion. The results were poorly reproducible, so an attempt to identify the products at the end of the experiments was made by ionophoresis of the hydrolysed material, in the experiment in which labelled substrate was used. Substances other than glucose and glucose phosphate were found to be formed, but were not further identified. This invalidated the results. The possibilities of this technique seem, from this experience, to be very restricted. In fact, in this case the presence, at a level of 1 part in 10 000, of a contaminating enzyme acting on either substrate would suffice to invalidate the results.

The effect of Mg^{2+} ion on FDNB inhibition suggests some sort of nitrogenous-group involvement in Mg^{2+}-ion binding, provided that the inactivation by FDNB is actually due to combination with a group involved in the binding of the metal. The protection by Mg^{2+} ion may be due to a prior combination with the group which reacts with FDNB. The effect in this case might be valuable in the identification of one of the groups involved in Mg^{2+}-ion binding and the amino acids around it.

However, that is not the only possible explanation of the effect of Mg^{2+} ion on FDNB inhibition. Consider the reaction sequence



Here the combination of Mg^{2+} ion with the enzyme, at a completely different site from the one that reacts with FDNB, should also lead to protection by Mg^{2+} ion, because the form E′ necessary for reaction with FDNB will be present in much smaller concentrations in the presence of Mg^{2+} ion.

On the other hand, the lack of an effective protection by magnesium during photo-oxidation does not exclude involvement of histidine, since the reactivity towards photo-oxidation of the imidazole ring may not be affected in such a way as to prevent the reaction, as is observed in the effect of Mg^{2+} ion on the inhibition by FDNB already discussed.

SUMMARY

1. Magnesium is an essential activator of phosphoglucomutase. However, in some cases some activity is found in its absence, which can be suppressed by ethylenediaminetetra-acetic acid and is probably due to residual traces of metals. The apparent Michaelis constant for magnesium is about 50 μM, with a concentration of glucose 1-phosphate of either 5 or 0·5 mM.

2. Magnesium forms complexes with both glucose 1-phosphate and glucose 6-phosphate, with dissociation constants slightly higher than 10 mM, and with the enzyme with a dissociation constant of about 0·5 mM.

3. Phosphoglucomutase is inhibited by 1-fluoro-2:4-dinitrobenzene, and magnesium protects against this inhibition at concentrations of the order required for full activation. The 1-fluoro-2:4-dinitrobenzene-inhibited enzyme has the same Michaelis constant for magnesium as the uninhibited enzyme. From hydrolysates of fully inactive dinitrophenyl-phosphoglucomutase, 6 molecules of dinitrophenyllysine and about 5 of dinitrophenyltyrosine per molecule of enzyme can be detected. All histidine residues have also reacted with the 1-fluoro-2:4-dinitrobenzene.

4. The activation by a 'second metal' in addition to Mg^{2+} ion is also found with pure phosphoglucomutase. It is suggested that its mechanism is similar to the activation by chelating agents.

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The Mechanism of Activation of Phosphoglucomutase by Chelating Agents

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Phosphoglucomutase, the enzyme that catalyses the interconversion of glucose 1-phosphate and glucose 6-phosphate, has a rather complex requirement for cofactors. The activation by metal ions was discussed in the preceding paper (Milstein, 1961*a*), and the effect of chelating agents is the subject of this one.

Several enzymes have been described as being activated by chelating agents. In some instances this activation is essential, i.e. the activity in the absence of the activator is negligible (see, for instance, Tabor & Mehler, 1955; Stoppani & Milstein, 1957; Najjar, 1948). Although removal of an inhibitory metal has been generally considered to be the mechanism of the activation, a binding of the chelating agent to the active site, as a necessary step in enzyme activation, has also been suggested (Mounter, Floyd & Chanutin, 1953; Sauer & Rapoport, 1959).

Lehmann (1939) first observed activation of phosphoglucomutase in crude extracts by glutathione, and Najjar (1948) found that the pure crystalline enzyme required cysteine for activity. Independently Jagannathan & Luck (1949) reported the activation of phosphoglucomutase by sulphite and serum albumin, and Sutherland (1949) reported activation also with histidine, free insulin, 8-hydroxyquinoline, dithizone and histamine.

This paper deals with the nature of the activation by chelating agents of phosphoglucomutase of rabbit skeletal muscle. It is shown that the absolute requirement for a chelating agent can be explained by an extreme sensitivity of the enzyme towards heavy metals, the concentration of which must be kept very low.

On addition of the enzyme to the assay mixture containing the chelating agent, removal of the metals and activation of the enzyme occurs. It will be shown that a different type of activation, which is time-dependent, also occurs when the enzyme is preincubated with either ethylenediaminetetra-acetic acid or Mg^{2+} ion plus a chelating agent.

A preliminary account of part of this work has been published (Milstein, 1960*a*).

MATERIALS AND METHODS

These were the same as described by Milstein (1961*a*), except the following:

'*Metal-free*' *phosphoglucomutase.* This was prepared by the following procedure: the purified enzyme was dialysed against large volumes of glass-distilled water and then

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against 1 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl, pH 7·5. It was then passed through an Amberlite IRC-50 column (8 cm. long) in the tris form, equilibrated with 1 mM-tris–HCl, pH 7·5. The metal content of the preparation after this treatment was undetectable by the 8-hydroxyquinoline test (Milstein, 1960*b*). The specific activity of the enzyme did not change with the treatment.

Special purification of the reagents. Metal-free tris–HCl buffer, pH 7·5, was prepared by passing the solution in the desired concentration through an Amberlite IRC-50 column treated with concentrated tris solution and then equilibrated with the dilute buffer solution.

The glucose 1-phosphate was freed from metal contamination before use by passing a solution of about 10 mg./ml. through an Amberlite IRC-120(H) column in the sodium or the potassium form. The potassium salt was generally used.

'Purified' glass-distilled water was obtained by passage through an Amberlite IR-120(H) column (30 cm. × 2 cm.) previously washed with 1N-HCl and then exhaustively with water.

When stated, $MgSO_4$ (A.R.) was also passed through an Amberlite IR-120(H) column which had been washed with HCl and then converted into the magnesium form with several volumes of 2M-$MgSO_4$. After washing the column with glass-distilled water, 6 mM-$MgSO_4$ was passed through.

Alternatively, a mixture of glucose 1-phosphate and Mg^{2+} ion, of the commercial (British Drug Houses Ltd.) potassium salt of glucose 1-phosphate and A.R. $MgSO_4$, in the appropriate concentrations at pH 7·5, was shaken with a solution of 0·01 % of dithizone in CCl_4 (Zn^{2+}-free, A.R.) twice to remove metals, and then (unless otherwise indicated) five times with CCl_4 to eliminate any trace of dithizone.

L-Cysteine–HCl was recrystallized from a constant-boiling HCl solution of the commercial (Roche) product. pH was adjusted to 7·5 with tris.

DL-Histidine–HCl was recrystallized from water and washed with acetone and ether.

RESULTS

That pure phosphoglucomutase from rabbit skeletal muscle is inactive when no metal-chelating agent is present in the assay mixture has already been shown (Najjar, 1948), and additional evidence is presented here. A comparison of the activating effect of different chelating agents is shown in Table 1. Qualitatively the activating effect can be related to the ratio of the binding capacity of the activator for heavy metals to that for magnesium. Thus cysteine, with a high affinity for heavy metals and a low one for magnesium, is the best activator. On the other hand, ethylenediaminetetra-acetic acid (EDTA), with a very high affinity for both, is a very poor activator, and at higher concentrations its inhibitory effect, due to removal of magnesium from the enzyme, becomes apparent.

Histidine is a good complexing reagent for heavy metals, but not for magnesium, and its activating effect approaches that of cysteine. EDTA and histidine activations are not additive (Table 1, Expts. C and D). The activation does not seem to

Table 1. *Effect of chelating agents on the activity of phosphoglucomutase*

The enzyme was incubated in the presence of 1·7 mM-$MgSO_4$, 7·5 mM-glucose 1-phosphate and the additions given.

Expt.	Enzyme (μg.)	Additions (mM)	Activity (μg. of P)	Relative activity (cysteine = 100)
A	1·4	28·6 Cysteine	56·8	100
A	1·4	0·074 EDTA	8·4	14·6
A	1·4	0·016 EDTA	12·8	22·5
A	1·4	0·0029 EDTA	10·8	19·0
A	1·4	0·0013 EDTA	7·4	13·0
A	1·4	0·0007 EDTA	2·6	4·6
A	1·4	None	1·3	2·3
B	2·0	5·7 Cysteine	39·4	—
B	2·0	2·8 EDTA	4·0	—
B	2·0	0·57 EDTA	15·8	—
B	2·0	0·057 EDTA	23·0	—
C	5·2	25·0 Histidine	40·3	—
C	5·2	25·0 Histidine + 0·12 EDTA	40·3	—
D	5·2	25·0 Histidine	46·5	—
D	5·2	25·0 Histidine + 0·25 EDTA	45·8	—
E	2·9	28·0 Cysteine	52·6	100
E	2·9	0·1 8-Hydroxyquinoline	23·4	44·5
F	1·7	19·0 Cysteine	40·2	100
F	1·7	4·2 8-Hydroxyquinoline	19·4	48·2
G	2·2	28·0 Cysteine	52·4	100
G	2·2	20·0 Na_2SO_3 + 0·4 % of egg albumin	15·0	28·6
H	Undetermined	20·0 Cysteine	17·8	100
H	Undetermined	22·0 Histidine	10·7	62
I	1·7	28·0 Cysteine	43·6	100
I	4·4	20·0 Na_2SO_3	4·6	4·2

depend on reduction, since sulphite either alone or with ovalbumin does not improve the activation by other chelating agents.

Metal content of phosphoglucomutase

Phosphoglucomutase dialysed against large volumes of ordinary glass-distilled water for 24 hr. contained metals. This was shown both by the 8-hydroxyquinoline test and by neutron-activation analysis.

Fig. 1 shows the effect of the addition of 8-hydroxyquinoline on the spectrum of the enzyme, with both water and 8-hydroxyquinoline as blanks (difference spectra). Subtraction of the protein spectrum from the difference spectrum yields the typical metal–8-hydroxyquinoline peak, showing the presence of metals in the preparation. The value of ΔE_{253}/mg. in this experiment is 0·560.

Neutron-activation analysis is an extremely sensitive method for metal determinations (Meinke, 1959; Loveridge & Smales, 1957). The results obtained after irradiation of phosphoglucomutase showed, besides the two characteristic peaks at 1·37 and 2·65 Mev with a half-life of roughly 14·5 hr. corresponding to ^{24}Na, another peak at 0·85 Mev which has a shorter half-life. It roughly corresponds with ^{56}Mn.

The radioactive material was allowed to stand over a period of 1 week until the short half-life radiation was insignificant, and the spectrum of radioactivity was again measured. Some radioactivity was still present, in the range 0·4–2·7 Mev, but no sharp peaks could be detected.

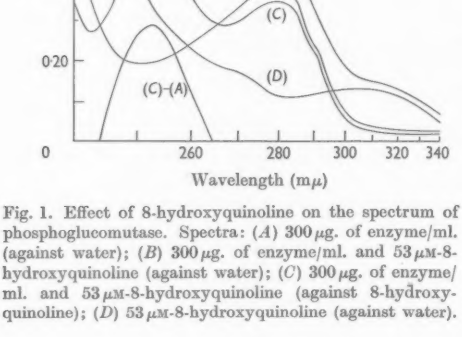

Fig. 1. Effect of 8-hydroxyquinoline on the spectrum of phosphoglucomutase. Spectra: (*A*) 300 μg. of enzyme/ml. (against water); (*B*) 300 μg. of enzyme/ml. and 53 μM-8-hydroxyquinoline (against water); (*C*) 300 μg. of enzyme/ml. and 53 μM-8-hydroxyquinoline (against 8-hydroxyquinoline); (*D*) 53 μM-8-hydroxyquinoline (against water).

The results of the activation analysis thus indicate the presence of a mixture of metals, each in small quantities, of which ^{56}Mn is the only one which could be identified, although some with long half-life are also present. The amount of manganese originally present is probably less than 0·3 atom/molecule of phosphoglucomutase, but this is only a tentative value calculated from the radioactivity measured (Loveridge & Smales, 1957) and is subject to errors, since no provisions for quantitative measurements were made. This amount of Mn^{2+} ion would not give a significant value in the 8-hydroxyquinoline test. It seems probable that the unidentified metals responsible for the long half-life radioactivity are also responsible for the high value of the 8-hydroxyquinoline test.

A 'metal-free' phosphoglucomutase was prepared as previously described. The 8-hydroxyquinoline test on the phosphoglucomutase solution at a concentration of 430 μg./ml. gave a reading at 253 mμ of 0·176. After the addition of the 8-hydroxyquinoline to both blank and sample the reading was 0·175. It is interesting that when this purified preparation with a ΔE_{253}/mg. value of 0·00 was dialysed for 24 hr. against glass-distilled water, the 8-hydroxyquinoline test rose again to a ΔE_{253}/mg. value of 0·810, suggesting that 'metal-free' phosphoglucomutase is able to bind the very low concentrations of metal impurities present in the glass-distilled water. The specific activity of the preparation did not change with the column treatment, showing that the removed metals are not necessary for activity.

When phosphoglucomutase was shaken with dithizone in CCl_4 the 8-hydroxyquinoline-test value was also lowered. The enzyme with an original ΔE_{253}/mg. value of 0·409 was treated as follows: a solution containing 264 μg./ml. of enzyme was shaken very gently for 30 sec. at 0°, with a 0·01% solution of dithizone in metal-free CCl_4, and the aqueous layer was separated after standing for some minutes. The 8-hydroxyquinoline test was then found to be negative. During dithizone treatment some denaturation occurs. Before the treatment the activity was 22·0 units/mg., and afterwards it was 18·4 units/mg., which represents a loss of 16% of the original value. This treatment also introduced traces of dithizone and CCl_4, judged by u.v. absorption. For freeing phosphoglucomutase from metals therefore the ion-exchange method was preferred.

Activation in the absence of chelating agents

'Metal-free' phosphoglucomutase had essentially the same requirements as the original enzyme. Removal of metals did not alter its specific activity, nor its requirement for chelating agents.

It has already been shown that in the absence of chelating agents the activity never reached values higher than 3% of the activity observed in the presence of 25 mM-cysteine. To increase this value, special purification of all the reagents was necessary.

All the reagents were purified with resin columns, as indicated in the Materials and Methods section, and special care was taken in the handling of the glassware (Thiers, 1957). After these precautions a several-fold increase in the activity in the absence of chelating agents was observed (Table 2). However, the values were still low compared with those obtained when cysteine was present. Values obtained in the absence of cysteine were poorly reproducible owing to the difficulty of preparing an absolutely 'metal-free' system. In the absence of cysteine the activity is adversely affected by using glass-distilled water instead of the 'metal-free' water, and by the use of $MgSO_4$ (A.R.) which was not especially purified.

Thus after the system has been freed from metals, chelating agents are no longer essential for activity. Another attempt to obtain full activity in the absence of chelating agents was made by treating the reagents with dithizone as follows.

A solution containing glucose 1-phosphate and $MgSO_4$ was shaken with 0·01% of dithizone in CCl_4 and the aqueous layer was separated after standing (first treatment). This solution was then shaken with CCl_4 to remove traces of dithizone and the aqueous layer was again separated after standing (second treatment). The activity of 2·1 μg. of 'metal-free' phosphoglucomutase was then measured with both treated solutions. A control with 11 mM-histidine as chelating agent was also used. The activity was 30·8 μg. of P converted in the histidine control, 25·7 when the supernatant from the first treatment was used (84·5% of control) and 22·3 when the supernatant from the second treatment was used (72·3% of control). The treatment with dithizone is thus very effective, although not as effective as histidine. Removal of the traces of dithizone slightly decreases the activity, but this difference is low, and indicates an inhibition due to the presence of minute amounts of metals in the CCl_4 rather than the diminution expected if the presence of dithizone in the reaction mixture was critical for activity.

When the mixture of glucose 1-phosphate and $MgSO_4$ was prepared as indicated above but then washed several times with CCl_4, the activity dropped to much lower values. After five washings (which may be considered sufficient to remove any possible traces of dithizone) the activity was 17% of the activity when cysteine was present as

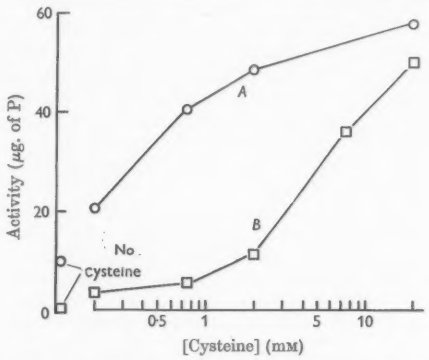

Fig. 2. Activation by cysteine in a 'metal-free' and a non-purified system. Activity is expressed as disappearance of acid-labile phosphate. ○, A mixture of glucose 1-phosphate and $MgSO_4$ purified by dithizone treatment, with cysteine added as indicated, together with metal-free tris–HCl to maintain ionic strength and pH. □, As before, but reagents were not 'metal-free'.

Table 2. *Activation of phosphoglucomutase by removal of metals*

The activity of 'metal-free' phosphoglucomutase was assayed in the usual way. Where indicated, 0·02 M-tris–chloride, pH 7·5, purified as described (Materials and Methods section) was used. Glucose 1-phosphate was in the Na form and purified as described. Where special purification is indicated, $MgSO_4$ and water were purified as described. Otherwise glass-distilled water and $MgSO_4$ (A.R.) were used.

Expt.	Enzyme (μg.)	Additions	Purity of $MgSO_4$ and water	P (μg.)	Activity (% of the complete system)
A	2·5	Complete system		63·8	100·0
A	5·5	No cysteine	—	5·2	3·8
A	5·5	No cysteine + tris–Cl	Purified	13·8	10·0
A	7·7	No cysteine	—	13·0	6·5
A	7·7	No cysteine	Purified	40·0	20·7
B	0·88	Complete system	—	14·2	100·0
B	2·2	No cysteine + tris–Cl	Purified	4·4	12·4
B	4·4	No cysteine + tris–Cl	Purified	23·8	32·2
B	9·9	No cysteine + tris–Cl	Purified	61·6	39·5
B	11·0	No cysteine + tris–Cl	Purified	61·8	34·8

activator. The activation with cysteine in such a purified system was studied quantitatively. It was observed to follow a different curve from that seen in a non-purified system (Fig. 2). The fact that the purified system requires less cysteine to activate the enzyme fully shows that activation involves removal of metals and not formation of a chelating-agent–enzyme complex.

Further activation by ethylenediaminetetra-acetic acid

In Table 1 it is shown that low concentrations of EDTA can partially replace cysteine, and that higher concentrations have an inhibitory effect. In these experiments the reaction was started by adding the enzyme to the reaction mixture containing all the components of the system, including the chelating agent. However, when the enzyme was previously incubated with EDTA and then assayed in the presence of cysteine by the usual procedure, a further increase in activity was observed, this being related to the time of preincubation (Fig. 3). The activation was complete after about 1 hr. of incubation. By this method, the specific activity of the enzyme increased to 60–64 units/mg. of protein.

Under these conditions the activation seems to be specific for EDTA, since other chelating agents such as cysteine, histidine, 8-hydroxyquinoline or tris–HCl buffer do not produce the same effect (Fig. 3). When cysteine was used in the assay mixture, the preincubation with EDTA usually increased the activity by a factor of 2·0–2·5. However, in some cases, especially when aged preparations of phosphoglucomutase were tested with histidine in the assay mixture, the activity was increased by as much as a factor of 6 (Table 3). The activation was also observed when 'metal-free' phosphoglucomutase was used.

When Mg^{2+} ion is added together with the chelate agent during the preincubation with phosphoglucomutase, activation with EDTA is still observed, but the effect is no longer specific for EDTA. Robinson & Najjar (1960) have recently reported preactivation by histidine or imidazole plus Mg^{2+} ion. That hydroxyquinoline is also effective is shown in Table 4.

The further activation by EDTA is reversible. Preactivated phosphoglucomutase drops to its original activity after exhaustive dialysis against tris buffer. This is best shown in the following experiment: 2 ml. of a solution containing 1 mg. of phosphoglucomutase was incubated for 75 min. at

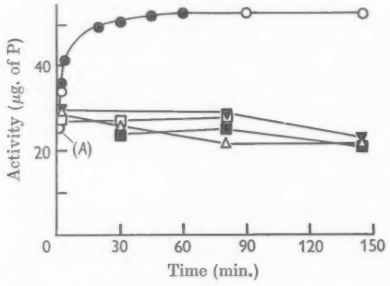

Fig. 3. Activation by preincubation with EDTA. Phosphoglucomutase (44·6 μg.) was incubated at 30° in the presence of 1 mM concentrations of the substances: ●, ○, EDTA (separate experiments); □, histidine; ■, 8-hydroxyquinoline; △, cysteine; ▼, tris. In all cases pH was adjusted to 7·5 with tris and the final volume was 1 ml. After the stated times 0·02 ml. was assayed for activity as indicated in the Materials and Methods section, with cysteine as activator and an incubation period of 2·5 min. Point (*A*) was determined with enzyme which was not preincubated with EDTA, in an assay mixture containing 50 μM-EDTA in addition to the other components.

Table 3. *Activation by preincubation with ethylenediaminetetra-acetic acid of 'metal-free' and aged phosphoglucomutase*

'Metal-free' phosphoglucomutase (Expt. A) or phosphoglucomutase 5 months old (Expt. B) was preincubated at 30° in the presence of 1 mM-EDTA, 10 mM-histidine and 1 mM-tris. The pH in all cases was 7·5. At different times, samples containing 1·3 μg. of phosphoglucomutase in Expt. A and 0·9 μg. in Expt. B were assayed for activity as indicated in the Materials and Methods section, with histidine present in the reaction mixture as activator.

Expt.	Time of preincubation (min.)	Specific activity of the enzyme (units/mg.) after preincubation with EDTA	Histidine	Tris
A	2	30·6	15·2	13·6
A	15	43·3	14·7	9·7
A	45	49·2	12·0	7·8
A	120	50·2	12·7	7·2
B	40	23·1	—	3·5

Table 4. *Activation of phosphoglucomutase by preincubation with 8-hydroxyquinoline in the presence of magnesium*

Phosphoglucomutase (30 μg.) was incubated at room temperature with 3 mM-$MgSO_4$ and 6·6 mM-8-hydroxyquinoline in 0·8 ml. final volume. Samples taken at different times were assayed in the presence of histidine.

Time of incubation (min.)	Relative activity (untreated enzyme = 100)
0·5	139
5	172
17	244
30	285

30° in the presence of 1 mM-EDTA, pH 7·5. The preparation was then dialysed for 24 hr. against 700 ml. of 1 mM-tris–HCl, pH 7·5, and again dialysed against a further 700 ml. for 48 hr. This preparation was diluted at 0°, and the activity tested at different times after the dilution. Under those conditions the activity quickly dropped to reach a constant value (Table 5).

The apparent Michaelis constant for Mg^{2+} ion does not seem to be affected by the EDTA pre-activation. This is shown in Fig. 4, for which an activated preparation dialysed 24 hr. against a large volume of tris–HCl, pH 7·5, was used.

The effect of EDTA on some physical properties of the enzyme was also investigated. The sedimentation pattern of the enzyme in 1 mM-EDTA is the same as when other media are used. The absorption spectrum of EDTA-activated phosphoglucomutase is essentially the same as the spectrum of untreated phosphoglucomutase. The ratio of the absorption at 220 and 278 mμ are, however, slightly different and at appropriate concentrations the curves cross at about 236 mμ.

Table 5. *Reversibility of the activation by preincubation with ethylenediaminetetra-acetic acid*

Phosphoglucomutase (496μg./ml. final concentration) was incubated for 75 min. at 30° in the presence of 1 mM-EDTA, pH 7·5. The enzyme was then dialysed against 350 times its volume of tris–HCl, pH 7·5; 0·04 ml. was then diluted 10 times with glass-distilled water, and 0·02 ml. samples were assayed for activity after different times of incubation at 0°. The assay was made as described in the Materials and Methods section with histidine as activator.

Time after dilution (min.) Dialysed enzyme	Activity (μg. of P)
0·5	27·2
1·5	17·1
8	12·7
25	12·6
Undialysed enzyme	40·0

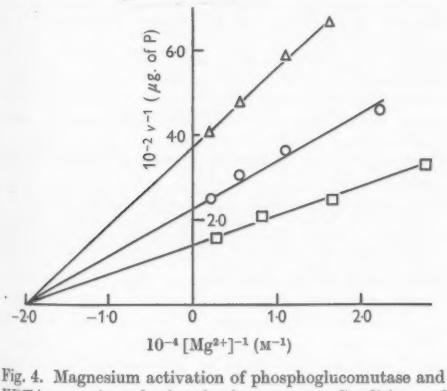

Fig. 4. Magnesium activation of phosphoglucomutase and EDTA-preactivated phosphoglucomutase. Conditions of assay were as described in the Materials and Methods section. Histidine was used as activator. △, 4·1 μg. of phosphoglucomutase; ○, 0·90 μg. of EDTA-activated phosphoglucomutase (dialysed once against mM-tris, pH 7·5); □, same as ○ but with 2·1 μg. of enzyme.

DISCUSSION

Chelating agents have two distinct activating effects on phosphoglucomutase of rabbit muscle. The first or 'essential' activation is obtained by including a chelating agent in the assay mixture. Negligible activity is obtained in its absence. This activation is due to removal of heavy metals, which are strong inhibitors of the reaction (Milstein, 1961*b*). However, if magnesium is also removed from the enzyme by excess of chelation, inhibition takes place. The second activating effect is obtained by preincubating the enzyme with EDTA [or, according to Robinson & Najjar (1960), with histidine plus magnesium]. In this case the activation is time-dependent and may produce a 2·5-fold increase in the molecular activity of the enzyme.

The evidence presented here led to the conclusion that crystalline phosphoglucomutase was in fact present as a metal–protein complex, even after exhaustive dialysis against glass-distilled water. It was therefore thought necessary to determine whether the active enzyme was necessarily a metal–protein or whether the essential activation by chelating agents really involved removal of inhibitory metals. That 8-hydroxyquinoline and EDTA, with higher affinity for metals than histidine, are less effective activators suggested, at first, that some mechanism other than removal of metals from the protein, such as a combination of the enzyme with the chelating agent, might be a possible explanation for the activation.

The fact that 'metal-free' phosphoglucomutase was fully active was not conclusive evidence against the hypothesis of the presence of a heavy metal at the active site, because of the possibility of reactivation by traces of metals in the test reagents. In fact, it was shown that 'metal-free' phosphoglucomutase was able to bind metals present in the distilled water. However, the fact that considerable activity (when tested in the absence of chelating agents) can be obtained by careful purification of the reaction mixture indicates that removal of inhibitory metals is the actual mechanism of the essential activation. Thus a satisfactory explanation for both activation and inhibition by metals (Milstein, 1961*a*, *b*) is the presence at the protein surface of a group or

groups with strong chelating properties, binding both Mg^{2+} ion and heavy metals. The best chelating activators are thus those removing heavy metals but not magnesium.

The mechanism of the second or 'further' activation obtained by preincubating phosphoglucomutase with EDTA is not clear at present. Robinson & Najjar (1960) published a preliminary communication on a further activation of phosphoglucomutase by incubation with histidine (which can be replaced by 8-hydroxyquinoline, as shown in this paper) plus magnesium. The extent of the activation, and the more marked response of aged preparations, are similar to those reported here with EDTA preactivation. The main difference in the results is that the activation as described by Robinson & Najjar requires both magnesium and histidine or imidazole. In the present work no added metal was required in the EDTA preactivation, and in fact 'metal-free' enzyme can also be further activated by EDTA. However, the possibility of minute contaminations with metal cannot be entirely ruled out at present, and considering the higher affinity of EDTA for several metals might provide an explanation for the apparent specificity of EDTA.

The second type of activation may possibly be explained by some change in protein configuration, which might be responsible for the small alteration in the absorption spectrum in the u.v. region. However, this is only tentative as little is known about the nature of the absorption at 220 mμ. The absorption at 280 mμ, on the other hand, is known to be affected by changes in the ionic state of tyrosine, connected with the tertiary structure of the protein.

SUMMARY

1. An 8-hydroxyquinoline test for metals has been used to show the presence of metals in the highly purified preparations of phosphoglucomutase, and to follow the preparation of a 'metal-free' enzyme. The 'metal-free' enzyme shows a positive test for metals after dialysis against glass-distilled water.

2. The necessity of a chelating agent in the assay of phosphoglucomutase is shown to be due to removal of inhibitory-metal impurities in the system. Considerable activity is obtained even in the absence of chelating agents if special precautions are taken to keep the system 'metal-free'. The extent of the activation by metal-chelating agents varies with the amount of the metal impurities present. It is concluded that the activation does not involve a complex of phosphoglucomutase and chelating agent.

3. If phosphoglucomutase is incubated with ethylenediaminetetra-acetic acid before the assay, a further activation of a different kind is obtained. This is time-dependent, reversible, not obtained with other chelating agents under the same conditions and does not affect the Michaelis constant for Mg^{2+} ion. If preincubation is made in the presence of magnesium, the further activation is also obtained with 8-hydroxyquinoline.

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Inhibition of Phosphoglucomutase by Trace Metals

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The interaction between phosphoglucomutase and metal ions is of great importance in the activation of the enzyme; the essential role of magnesium has already been discussed (Milstein, 1961*a*). In addition to magnesium, a chelating agent is also essential for activity (Najjar, 1948; Sutherland, 1949). The necessity for chelating agents is due to the removal of trace metals present in the assay mixture, which are inhibitory to the enzyme (Milstein, 1960, 1961*b*). Additional evidence for this view has now been obtained in a quantitative study of the inhibition of phosphoglucomutase by Zn^{2+} and Cu^{2+} ions.

Phosphoglucomutase is very sensitive to heavy metals. Copper and Hg^{2+} ions (Jagannathan & Luck, 1949), or Zn^{2+}, Cu^{2+}, Ag^{+} and Hg^{2+} ions (Sutherland, 1949), inhibit the rabbit-muscle and yeast enzymes respectively. Recently, however, McCoy & Najjar (1959) reported that Zn^{2+} ions could replace Mg^{2+} ions as activator for the yeast enzyme.

Since phosphoglucomutase has been listed as a 'SH enzyme' (Barron, 1951) it was thought that the inhibition might be a result of blocking by the metal of the SH groups essential for activity. The evidence for the presence of active SH groups in the enzyme was, however, rather poor. Only scattered studies have been reported in the literature. Lehmann (1939) found inhibition of crude muscle preparations by oxidized glutathione. With crude yeast preparations incubation with 2 mM-iodoacetate for 10 min. produced 50% inactivation (Sutherland, 1949). Iodoacetate, however, is one of the most unreliable of SH inhibitors, both for its lack of specificity and for its failure to react at low concentrations (Dixon, 1948; Barron, 1951). Furthermore, the experiments of Lehmann were made on very crude preparations, for which the cofactor requirements were still unknown. It is shown in this paper that there are no grounds for considering phosphoglucomutase as an enzyme depending on its SH groups for activity. The high affinity of the enzyme for heavy metals at its active site is probably concerned with other groups.

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MATERIALS AND METHODS

Abbreviations used are: tris, 2-amino-2-hydroxymethylpropane-1:3-diol; pCMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetra-acetic acid.

Materials and methods were as described in previous papers (Milstein, 1961*a*, *b*), except for the following:

Thiol groups. These were titrated with pCMB by the method of Boyer as described by Fraenkel-Conrat (1957), modified for use with a 1 ml. microcell. Readings in the ultraviolet were made in a Unicam SP. 500 spectrophotometer.

Inhibition studies. Inhibition studies with metals were made with the complete system, and the inhibition values were referred to the concentration of free metal present. For a quantitative study of the inhibition, the 'metal-buffer' technique (Raaflaub, 1956) was used. By this technique the concentration of free metals is kept at known low values by the use of chelating agents. A histidine 'metal buffer' was used and the concentration of the free metal was altered by changing the concentration of either total metal or histidine. The enzyme activity was then determined with different concentrations of the free metal, and expressed as a percentage of the activity when no metal was added apart from Mg^{2+} ion, which was present in optimum concentration.

With the SH reagents, the buffered enzyme (30–80 μg./ml.) was incubated with the inhibitor and samples (0·02–0·05 ml.) were taken at different times and assayed for activity. Protection by components of the assay system is thus avoided. The inhibition is expressed as

$$I\,(\%) = (1 - A/A_0) \times 100,$$

where A is the activity of the inhibited enzyme and A_0 the activity of a control treated in the same way but without inhibitor.

RESULTS

Inhibition by Zn^{2+} *and* Cu^{2+} *ions*

Zinc and Cu^{2+} ions are powerful inhibitors of muscle phosphoglucomutase. An experiment with Zn^{2+} ions is shown in Fig. 1. The activities in the presence of different concentrations of Zn^{2+} ions and histidine are plotted against the concentration of free Zn^{2+} ions, calculated by the method of Raaflaub (1956), the dissociation constant of the Zn–histidine complex and the pK of the groups involved being obtained from tables. Inhibition of 50% is obtained at a concentration of free Zn^{2+} ion $1{\cdot}7 \times 10^{-12}$ M.

A similar experiment with Cu^{2+} ions is shown in Fig. 2, in which the relative activity is plotted against concentration of free Cu^{2+} ions, determined by the same method. The curve in this case is much steeper at the mid-point, and in fact suggests that more than one complex is involved. Theoretical calculations for complexes of 2 or 3 atoms of copper/molecule of enzyme, each inactive complex being formed independently of the other(s), did not fit the experimental points exactly, although they were much closer than that for only one inactive complex. The best fit is obtained for between 2 and 3 atoms/molecule of enzyme. The concentration of free Cu^{2+} ions giving 50 % inhibition is $3{\cdot}8 \times 10^{-16}$ M.

Since Mg^{2+} ion is an essential activator of the enzyme reaction, it was present in a concentration of 1·5 mM in the experiments mentioned above. The relationship between Mg^{2+} ion activation and heavy-metal inhibition was investigated by studying reciprocal plots of activity at different Mg^{2+} ion concentrations in the presence and the absence of inhibitory metal. The effect of Zn^{2+} ion follows the pattern of a purely competitive inhibition (Fig. 3). The dissociation constant for the inhibitor can be calculated, since the effect of the inhibitor will be to produce an increase in the apparent Michaelis constant for the activator ($K_{app.}$) by a factor of $1+i/K_i$ (i being the concentration of inhibitor and K_i its dissociation constant), as with inhibition due to competition with the substrate (Malmström, 1955). From Fig. 3:

$$\frac{\text{Slope } A}{\text{Slope } B} = 1+\frac{[\text{Free } Zn^{2+} \text{ ions}]}{K_{Zn}}. \qquad (1)$$

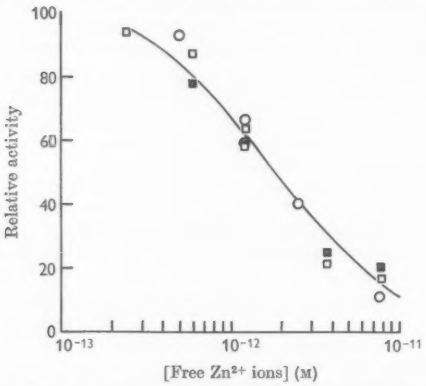

Fig. 1. Inhibition of phosphoglucomutase reaction by Zn^{2+} ions. The activity of 7 μg. (open symbols) or 3 μg. (solid symbols) of metal-free phosphoglucomutase was determined under standard conditions (incubation periods of 4 and 2 min. respectively), except that histidine was used in concentrations from 35 mM to 1·2 mM, and $ZnSO_4$ was present in concentrations of 0·175 mM (○, ●) and 0·087 mM (□, ■).

Relative activity is expressed as percentage of the activity in the absence of added Zn^{2+} ions. In the calculation of the amount of free Zn^{2+} ions, log $K^{Zn}_{ZnHis} = 12{\cdot}0$.

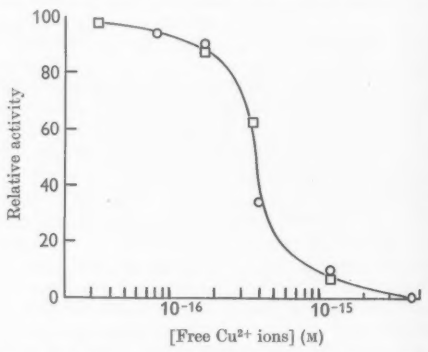

Fig. 2. Inhibition of phosphoglucomutase reaction by Cu^{2+} ions. The activity of 6 μg. of 'metal-free' phosphoglucomutase was determined under standard conditions (incubation period 2·5 min.), except that histidine was used in concentrations of 25 mM (□) or 2·5 mM (○), and $CuSO_4$ added in concentrations from 3·2 mM to 0·32 mM. Activity is expressed as percentage of the activity in the absence of added Cu^{2+} ions. The value log $K^{Cu}_{CuHis} = -18{\cdot}3$ (Maley & Mellor, 1950) was used for the calculation of [free Cu^{2+} ions].

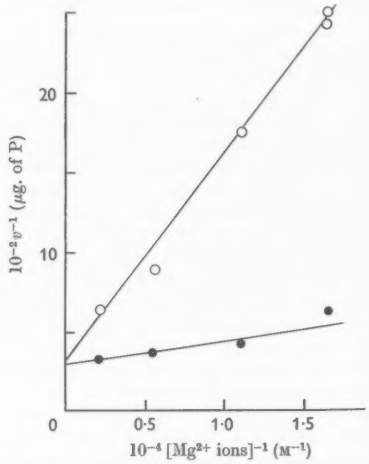

Fig. 3. Effect of Zn^{2+} ions on Mg^{2+} ion activation. Conditions were as in the standard assay. Histidine 25 mM; 4·1 μg. of phosphoglucomutase. A (○), [Free Zn^{2+} ions] = $3{\cdot}2 \times 10^{-13}$ M, $ZnSO_4$, $1{\cdot}3 \times 10^{-4}$ M; B(●), no addition.

The value of the inhibitor constant, K_{Zn}, calculated in this way is $3{\cdot}9 \times 10^{-14}$ M. With yeast phosphoglucomutase, Zn^{2+} ion can partially replace Mg^{2+} ion as an activator (McCoy & Najjar, 1959). Since the coenzyme and substrate requirements and the equilibrium constant of the two steps of the reaction are the same for both enzymes, this difference between the yeast and muscle enzymes in the effect of Zn^{2+} ions may perhaps be ascribed to different enzymic configurations. This again points to the importance of the enzyme–metal complex in the activation, as discussed by Milstein (1961*a*).

Copper ion, on the other hand, is not a purely competitive inhibitor. This is shown in Fig. 4, which again suggests a participation of more than one atom of copper/enzyme molecule at the range of concentration used. As can be seen, both $K_{app.}$ (apparent Michaelis constant of the activator) and V are affected by the inhibition. Malmström (1955) derived an equation for the enolase system, assuming that one molecule of inhibitor I (with a dissociation constant K_i) competes with the activating metal (m) and that another molecule of I (with a dissociation constant K'_i) binds the enzyme, giving an independent non-competitive inhibition.

$$v = \frac{VK'_i m}{[K_{app.}(1+i/K_i)+m]\,(K'_i+i)}. \qquad (2)$$

Then
$$vc = \frac{V}{1+K_{app.}/m[1+(i/K_i)]}, \qquad (3)$$

where
$$c = 1+i/K' \qquad (4)$$

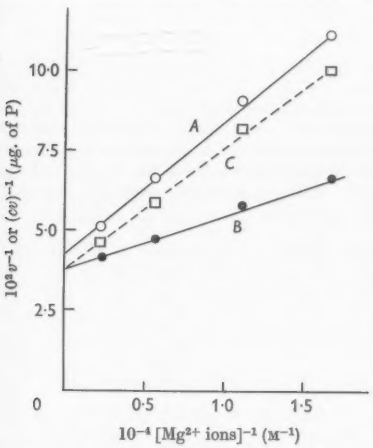

Fig. 4. Effect of Cu^{2+} ions on Mg^{2+} ion activation. Conditions were as in the standard assay. Histidine, 25 mM; 4·1 μg. of phosphoglucomutase. *A*, [Free Cu^{2+} ions] = $2{\cdot}5 \times 10^{-17}$ M, $CuSO_4$, 1·2 mM; *B*, no addition; *C*, as *A* but the ordinate is $(cv)^{-1}$, with $c = 1{\cdot}1$. (For explanation see text.)

c may be calculated from the values of *V*, obtained by plotting $1/v$ against $1/m$ in the presence and the absence of inhibitor [V_B and V_A respectively (see Fig. 4)]. At infinite concentration of m, $V_B = V_A c$ (see equation 3). Now, replacing the ordinate $1/v$ by $1/cv$ the new line should resemble that produced by a competitive inhibition (Fig. 4, line *C*). The two dissociation constants can thus be calculated: K_i, from the slopes of lines *B* and *C* (Fig. 4), with a value of $2{\cdot}3 \times 10^{-17}$, and K'_i, from equation (4), with a value of $2{\cdot}5 \times 10^{-16}$. These values suggest that, at low concentrations of Mg^{2+} ion, Cu^{2+} ion inhibition is mainly due to the formation of a complex with the Cu^{2+} ion at the same site as the active Mg^{2+} ion, but as the concentration of Mg^{2+} ion increases, higher concentrations of Cu^{2+} ion are necessary for inhibition and Cu^{2+} ion then begins to combine non-competitively with other sites. The agreement between the experimental data in Fig. 4 and the assumption of two complexes must be treated with some caution, mainly because of the fact that the inhibition by formation of the second complex is very small and a more complicated effect might be present, although not evident under these conditions.

Thiol groups in phosphoglucomutase

A number of reagents that give an irreversible inhibition with enzymes containing essential SH groups for activity were tested, including an oxidizing reagent (*o*-iodosobenzoate), two alkylating reagents (chloroacetophenone and iodoacetate) and an unsaturated imide (*N*-ethylmaleimide).

Iodosobenzoate in low concentrations usually requires only a few minutes to react with SH groups. To obtain inhibition with phosphoglucomutase, rather long incubation periods and high concentrations of the reagents were necessary. Thus as is shown in Table 1, Expt. A, 0·4 mM-iodosobenzoate (which represents a 1000-fold excess compared with the amount of enzyme) requires 10 min. incubation for a detectable inhibition. It may then be concluded that the results do not support the idea of specific oxidation of SH groups. 'Metal-free' phosphoglucomutase reacts very much like phosphoglucomutase (Table 1, Expt. B).

The effect of *N*-ethylmaleimide on free SH groups is very rapid (Gregory, 1955). Concentrations of 1 μM have an antimitotic effect (Friedman, Marrian & Simon-Reuss, 1949), and unequivocal SH inhibitions at low enzyme concentrations are usually complete with 10 μM-*N*-ethylmaleimide in less than 5 min. This is not the case with phosphoglucomutase. The reaction is very slow indeed, and even high concentrations fail to inactivate the enzyme significantly after short periods of incuba-

Table 1. *Effect of sulphydryl inhibitors on activity of phosphoglucomutase*

'Metal-free' phosphoglucomutase was used in Expts. B and C. Phosphoglucomutase dialysed overnight against 1 mM-EDTA was used in Expt. E. Other conditions were as indicated in the Materials and Methods section.

Expt.	Addition	Buffer	Time (min.)	Inhibition (%)
A	Iodosobenzoate 0·02 mM	0·1M-Sodium acetate	10	1·0
	0·4 mM		10	11·8
	2·0 mM		10	21·5
B	Iodosobenzoate 1·7 mM	58 mM-Histidine, pH 7·5	25	27·3
C	*N*-Ethylmaleimide 5·0 mM	58 mM-Histidine, pH 7·5	25	48·3
D	Chloroacetophenone 2·0 mM	40 mM-Sodium acetate	10	7·5
	2·0 mM		70	29·0
	2·0 mM		150	68·0
E	Chloroacetophenone 2·0 mM	40 mM-Sodium acetate	10	−3·0
	2·0 mM		55	−9·0
	2·0 mM		125	9·0
	2·0 mM		220	33·4
F	Iodoacetate 2·0 mM	10 mM-Sodium bicarbonate	25	33·3
	2·0 mM	20 mM-Acetate buffer, pH 5·7	25	0·3
G	Oxidized glutathione 20·0 mM	0·1 M-Tris–HCl, pH 7·5	15	25·0

tion. However, a slow reaction takes place, which in 2 hr. produces a complete inactivation. Table 1 (Expt. C) shows an experiment in which about 50 % inhibition was obtained with 5 mM-*N*-ethylmaleimide and 'metal-free' enzyme after 25 min. incubation.

Alkylating agents are in general unreliable for the detection of SH groups (Barron, 1951). Chloroacetophenone, however, seems to be a reasonable one to choose since it has been shown to react slowly with native ovalbumin, and very fast (when slight excess is present) with urea-treated ovalbumin (Dixon, 1948). 2 mM-Chloroacetophenone, representing a 2000-fold excess of the reagent over the enzyme, produces 50 % inhibition after 2 hr. incubation (Table 1, Expt. D). The reaction with an EDTA-dialysed enzyme is even less pronounced.

As mentioned above, iodoacetate is the most unreliable of all the so-called SH reagents. Even in cases in which other SH reagents are potent inhibitors its reaction is often very slow, and it is unspecific since it also reacts with histidine, lysine and methionine (Gundlach, Stein & Moore, 1959). The effect of this reagent on phosphoglucomutase follows a pattern similar to that of other SH inhibitors. High concentrations and very long incubation periods are necessary for inhibition. The results may indicate a reaction with an amino group of lysine since inhibition is not observed at slightly acidic pH values. The fact that no inactivation is observed at pH 5·7 seems to indicate that if a histidine residue is essential for catalysis, it does not react with iodoacetate under these conditions.

Oxidized glutathione has also some inhibitory effect (Table 1, Expt. G).

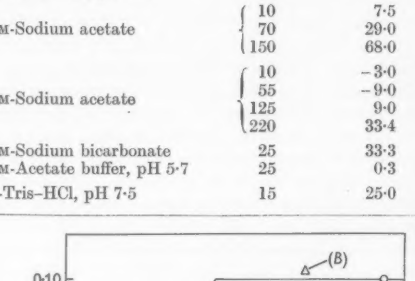

Fig. 5. Titration of SH groups with pCMB. Phosphoglucomutase (6·5 μM) in: △, 0·01 M-phosphate buffer, pH 7·0; ○, 12 mM-histidine buffer, pH 7·5; □, 12 mM-histidine buffer, pH 7·5, +44 μM-$ZnSO_4$. pCMB was added with a micropipette to both blank and sample. The lines indicate the theoretical titration curve for 1 and 2 SH groups/molecule. Point (*B*) is a second reading of point (*A*) after 15 min. incubation.

When phosphoglucomutase is titrated for SH groups with pCMB, about two SH groups/molecule are detected, and these react quite fast with the reagent, producing the characteristic shift in the absorption at 250 mμ at pH 7·0. The titration is the same when histidine buffer is used instead of phosphate. The presence of 44 μM-$ZnSO_4$ together with the histidine does not introduce any significant change (Fig. 5). The addition of 1·7 mM-Zn^{2+} ions to the cuvette containing phosphate buffer (see Fig. 5) after two SH groups have been titrated does not produce any decrease in extinction. The same is true after addition of 0·14 mM-$ZnSO_4$ to the cuvette containing histidine.

On leaving excess of pCMB in contact with the enzyme in phosphate buffer (with or without 1·7 mM-$ZnSO_4$) for 2 hr., an increase in ΔE_{250} is produced which corresponds to a titration of three SH groups in all. Higher values have also been obtained on longer incubations.

The material after exposure to pCMB for varying times was assayed for activity by incubating a sample of the solution with the standard assay mixture containing histidine as activator. After titration of two SH groups, 75% of the enzyme activity remains, and after three SH groups have reacted the activity falls to about 50% of its original value. The activity is restored by incubating the enzyme with 0·1 M-cysteine, but incubation for 15 min. is required.

The result shows that a reaction takes place with the enzyme which partially inactivates the enzyme and is not immediately reversed by cysteine, as would be expected if the loss of activity were due to a simple reaction of pCMB with SH.

DISCUSSION

The mechanism of activation of phosphoglucomutase by chelating agents proposed in the preceding paper (Milstein, 1961*b*) is based on the breaking down of highly stable inactive metal–enzyme complexes. The complexes described here have these properties. The reason for using Zn^{2+} and Cu^{2+} ions in the studies is that they are common contaminants, especially in distilled water. The concentrations of these metals even in glass-distilled water may easily reach the values necessary for complete inactivation of phosphoglucomutase, especially if the still is not carefully designed (Thiers, 1957).

The extremely low value of the dissociation constants for the inhibitory Cu^{2+} and Zn^{2+} ions poses the question whether they have any physiological significance. This will in fact depend on the actual concentration of free metal ions in the tissue. If these concentrations are higher than the concentrations required for inhibition (10^{-16} M for Cu^{2+} ions), the activation by chelating agents may be a mechanism of control of glycolysis by hormones or other natural chelating agents. It is possible, however, that the activation is one of the artifacts of testing of activity *in vitro* arising from the extraction of the enzyme from its natural environment.

The effect of SH inhibitors on phosphoglucomutase gives some indication that destruction of SH groups may produce a partial loss of activity. This is suggested by the fact that inhibitors such as *N*-ethylmaleimide, iodosobenzoate and oxidized glutathione can partially inactivate the enzyme. However, the participation of SH groups in these reactions is very uncertain. Under the conditions in which inhibition occurs, secondary reactions are likely to take place. With high concentrations of iodosobenzoate breaking of –S–S–bonds and reaction with other groups such as hydroxyl or amino groups would be expected (Rabinovitch & Barron, 1955). A good example is the inhibition of ribonuclease by iodosobenzoate, interpreted by Ledoux (1954) as an effect on SH groups; it is now known that ribonuclease does not contain free SH groups. With excess of *o*-iodosobenzoate protein denaturation may be produced, as shown by Hellerman, Chinard & Deitz (1943) for urease. Lack of reaction of *N*-ethylmaleimide with –S–S– has been reported by Gregory (1955). However, recently a reaction of *N*-ethylmaleimide with amino groups and with imidazole has been described by Smyth, Nagamatsu & Fruton (1960). The inhibition obtained with pCMB is not instantaneously reversed by high concentrations of cysteine; incubation for 15 min. is required for full recovery. The results suggest that the rate-limiting step is not the reaction between the inhibitor and a SH group but perhaps some type of unfolding of the protein molecule. Unspecific reactions are, however, possible with any of the reagents. The results do not support the idea that a SH group is directly involved in the catalytic action of the enzyme.

The lack of specific inhibition of the enzyme by SH reagents is an indication that its sensitivity towards heavy metals is not due to combination of the metals with SH groups. Furthermore, the fact that Zn^{2+} ion did not affect either the titration of SH groups or the partial inactivation of the enzyme by pCMB is a further indication that the high affinity of the enzyme for Zn^{2+} ion involves groups other than SH. This high affinity can be explained by the assumption that more than one side chain of the protein is involved in the chelation (Barnard & Stein, 1958; Malmström & Rosenberg, 1959). An alternative possibility would be combination with a *N*-terminal histidine residue, which is known to have a very high affinity for metals (Barnard & Stein, 1958), but even this affinity would not be high enough to explain the fact that a 10^4–10^5 excess of histidine with respect to enzyme is necessary for full activity (Milstein, 1961*b*).

Since SH groups may be excluded, a chelating site including amino, imidazolyl, carboxyl or phosphate groups may be involved. A pentapeptide with the sequence threonylalanylphosphoserylhistidylaspartic (or asparagine) (Thr.Ala.SerP.His.Asp) has recently been shown to be present in the active centre of phosphoglucomutase by Milstein & Sanger (1961). In a three-dimensional model of the peptide, arranged as in an α-helix, the imidazole ring is free to move so as to occupy a position very close to the phosphate or to the carboxyl

group of aspartic acid, giving two configurations, both of which could be expected to have strong metal-chelating properties.

SUMMARY

1. A quantitative study of the inhibition of phosphoglucomutase provides additional evidence that the necessity for a chelating agent is due to the presence of minute traces of inhibitory metals.

2. Zinc is an inhibitor of phosphoglucomutase, competing with Mg^{2+} ions, with dissociation constant K_{Zn} $3{\cdot}9 \times 10^{-14}$M. Copper also competes with Mg^{2+} ions, but the inhibition is not purely competitive. The inhibition by Cu^{2+} ions can be explained by competition with the Mg^{2+} ions (K_{Cu} $2{\cdot}3 \times 10^{-17}$M), together with non-competitive combination at a second site (K'_{Cu} $2{\cdot}5 \times 10^{-16}$M).

3. Three to four SH groups are present in phosphoglucomutase. Although some inhibition is observed with high concentrations of several SH reagents, phosphoglucomutase does not appear to be an enzyme requiring these groups for activity.

4. The effects are discussed in the light of the amino acid sequence in the active centre previously determined.

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The Effect of Insulin on Amino Acid Metabolism

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In the course of studies on nitrogen metabolism in the alligator, it was observed that glycine, alanine, glutamine and leucine were the main amino acids utilized for the renal synthesis of ammonia (Coulson & Hernandez, 1959). The injection of insulin caused a dramatic decrease in the plasma concentrations of these compounds and as a consequence the renal synthesis of ammonia was virtually abolished.

The knowledge that plasma amino acids will decrease after the injection of insulin is almost as old as the observation that insulin lowers blood glucose (Luck, Morrison & Wilbur, 1928). For several reasons, however, there have been few studies of the effect of insulin on individual amino acids of either configuration. Only recently have the pure isomers been available at a reasonable cost, and even now the amount required for experiments on large mammals such as the dog makes such studies exceedingly expensive. When an amino acid is given to a mammal by any route the compound is so quickly absorbed and metabolized or excreted that studies *in vivo* are often impractical. In spite of these limitations Hier (1947) compared the 'tolerances' of several amino acids in the dog. No plasma rise occurred after the oral admini-

stration of DL-tryptophan, L-cystine, DL-phenylalanine and L-tyrosine and very little rise followed the ingestion of L-leucine, DL-isoleucine and L-arginine. DL-Threonine, DL-valine and L-histidine increased the plasma amino acid levels appreciably for a short time and only DL-methionine remained in the plasma in considerable amounts for a period of several hours. The effects of insulin were not studied.

The alligator with a metabolic rate of about 5% of that of man offered an opportunity to study not only the rates at which the individual D- or L-amino acids disappeared from the plasma but also the influence of insulin on those rates. Reactions which are completed in minutes in a mammal last for days or even weeks in the alligator and since the reactions are slow, the quantities of amino acids needed are much less than for comparable studies in mammals. In an effort to determine whether information derived from the alligator experiments could be applied to a mammal, amino acid tolerances were also performed on rats.

It has been suggested that insulin exerts its effects on amino acids by catalysing their conversion into proteins (Lotspeich, 1949; Russell, 1955; Frame & Russell, 1946). The results of the present study suggest that its action is more involved than this.

MATERIALS AND METHODS

Animals. The 200 alligators (*A. mississippiensis*) used in these studies were bought from dealers who collected them from the swamps of Louisiana. They were kept in large concrete tanks with water in a temperature-controlled room (28°). Under laboratory conditions they ate well and were easy to handle. They were fed chopped rats or rabbits once or twice a week. Before each experiment food was denied for at least a week after which time the plasma amino acid level remained fairly constant at about 2-4 m-moles/l. In the majority of experiments animals weighing about 1 kg. were used, but in those experiments with the more expensive and rare amino acids it was necessary to use animals weighing as little as 100 g. In each amino acid tolerance experiment the group receiving insulin was paired with controls of the same weight. Over the period of many months covered by these studies, many of the alligators were used several times since they were not killed in the experiments.

The rats were young adults of both sexes of the Sprague–Dawley strain weighing 100–200 g. They were kept on Purina Laboratory Chow manufactured by Ralston Purina Co., St Louis, Mo.

Blood samples. Blood for the various analyses was obtained by cutting the tip ends of the tails of both the alligators and the rats and gently milking about 0·2–0·8 ml. of blood into heparinized capillary tubes. When larger amounts of blood were required as much as 5 ml. were withdrawn from the heart. The blood was centrifuged and the plasma was separated from the cells and stored in the freezing compartment of a refrigerator until analysed.

In the experiments with rats, blood was removed at about 15 min. intervals after the injection of the amino acids, and this was continued until the plasma levels had returned to the control values. Owing to the very low metabolic rate of the alligator, blood was obtained at less frequent intervals ranging from 2 to 24 hr., depending upon the rate at which the amino acids were utilized.

For the amino acid analyses protein-free filtrates were prepared by adding 4 vol. of ammonia-free ethanol to plasma in small test tubes. These were then centrifuged and the protein-free supernatants were separated. Samples were used for the various amino acid analyses.

Injections. The insulin used was Iletin, the crystalline zinc insulin of Eli Lilly and Co. The alligators were injected intramuscularly with 100 units/kg. body wt. 1–2 days before the amino acid to be tested to insure a maximum physiological effect. Such a dose was effective for a full 10 days, and after the third day it was necessary to give glucose to prevent hypoglycaemia. The effect of insulin on the rate of amino acid utilization in the rat was not studied.

The amino acids were obtained from Nutritional Biochemicals Inc., Cleveland, Ohio, and in those few instances in which impurities were suspected they were recrystallized before use. Although several experiments on both alligators and rats were performed in which the amino acids were injected intracardially or given by stomach tube, the most consistent results were obtained after the intraperitoneal injection of 10 m-moles/kg. body wt. of an iso-osmotic solution for the soluble compounds, and as an equivalent saturated solution for such sparingly soluble amino acids as leucine, isoleucine, tryptophan and phenylalanine. Aspartic and glutamic acid were injected in the form of their monosodium salts and arginine, histidine, lysine and ornithine in the form of their monohydrochlorides.

Amino acid and glucose analyses. The non-protein amino acid composition of alligator plasma was determined by the microbiological method of Steel, Sauberlich, Reynolds & Baumann (1949) and Sauberlich & Baumann (1949) with the exception of glutamine, which was estimated by the glutaminase method of Iber & Bruton (1956).

Depending on the amino acid to be measured microbiologically and the quantity known to be required for optimum growth (Steele *et al.* 1949), 0·05–2·0 ml. of the alcoholic 1:5 protein-free plasma filtrate was put in a culture tube and the alcohol was evaporated in a water bath. Sterile water (1 ml.) and sterile medium (1 ml.) were added and each tube was inoculated with 0·001 ml. of a washed suspension of organisms in 0·9% NaCl solution. The culture tubes were incubated for 3 days at 38° and the lactic acid formed was estimated by titration. The titration values were compared with those obtained with a series of tubes which contained standard amounts of the amino acid to be measured.

In the amino acid tolerance experiments the high plasma concentration of the injected amino acid made it easy to estimate the amino acid microbiologically with accuracy. Plasma from untreated alligators deprived of food for two weeks, however, contained only traces of several of the amino acids and some difficulty was encountered in the estimation of those present in very low amounts. Glutamic acid, in particular, required as much as 2 ml. of plasma to give enough of the protein-free filtrate for satisfactory bacterial growth.

The ninhydrin method of Troll & Cannan (1953) was used for the determination of total amino acids in the amino acid tolerance experiments. A routine was adopted in

Table 1. *Amino acid composition of alligator and human plasma expressed in terms of per cent of the total amino acids determined by microbiological analysis*

'Total amino acids' in this table refers only to the sum of the 18 amino acids which were identified and analysed by microbiological assay. Since only about three-quarters of the total α-amino acids were accounted for in the microbiological assays, the percentage values in the table represent a slight exaggeration. The values for man were calculated from the data of Harper, Hutchin & Kimmel (1952), and those for the alligator represent the values obtained from twelve alligators which had been starved for 2 weeks. The 'total amino acid' concentration of alligator plasma was 1·68 (2·05 by ninhydrin) and that for man was 3·70 mM (ninhydrin total not reported). For abbreviations of amino acids see *Biochem. J.* (1957), **66**, 6. Tr, trace.

	Alligator		Man			Alligator		Man	
Amino acid	Mean (%)	Range (%)	Mean (%)	Range (%)	Amino acid	Mean (%)	Range (%)	Mean (%)	Range (%)
Gly	27	(12–32)	11	(3–20)	Thr	4	(2–7)	5	(2–9)
Ala	12	(5–16)	12	(7–23)	Ileu	2	(1–4)	4	(2–8)
Glu-NH_2	12	(5–30)	14	(9–20)	Cy_2S_2	2	(1–3)	7	(4–11)
Val	8	(7–9)	8	(6–10)	Lys	2	(0–3)	7	(4–11)
Leu	7	(3–14)	5	(2–10)	His	1	(0–2)	4	(4–7)
Ser	6	(5–7)	4	(1–6)	Try	1	(0–2)	2	(1–3)
Pro	5	(0–6)	6	(3–13)	Arg	1	(0–2)	4	(2–5)
Phe	5	(4–7)	4	(2–8)	Met	0·6	(0–0·8)	1	(0·5–2)
Glu	4	(0–8)	2	(0–3)	Tyr	0·6	(0–0·7)	2	(2–4)
					Asp	—	—	Tr	(0–Tr)

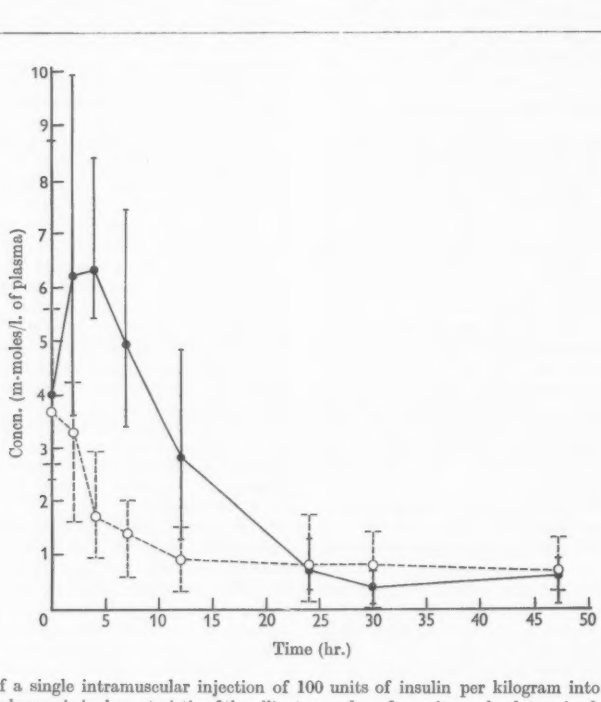

Fig. 1. Effect of a single intramuscular injection of 100 units of insulin per kilogram into small alligators. The initial hyperglycaemia is characteristic of the alligator, and, so far as it can be determined, this effect is not due to the contamination of insulin with glucagon. The hypoglycaemia and hypoaminoacidaemia persisted for about 10 days. The perpendicular lines indicate the ranges observed in 10 experimental animals. ——, Plasma glucose; – – – plasma amino acids.

which the total amino acid concentration was estimated by the ninhydrin method, and occasional samples in each experiment were analysed by microbiological analyses. By this means it was possible to verify the fact that, with rare exceptions, the rise in total plasma amino acids after an injection was due almost entirely to an increase in the amino acid injected. For proline the curves are based solely on microbiological analyses. No satisfactory method was available for the specific analysis of the D-isomers and the tolerance curves reported were derived entirely from the ninhydrin analyses.

Plasma glucose of the alligator was estimated by the glucose oxidase method of Keston (1956) with the commercial reagents and the modifications recommended by the Worthington Biochemical Corp., Freehold, N.J. This very convenient procedure required only 0·1 ml. of plasma. When it was discovered that the method of Folin & Wu (1920) gave identical results for the alligator, this method was then employed instead.

RESULTS

Amino acid composition of alligator plasma

As a preliminary to the proposed tolerance tests 9 ml. of plasma from each of 12 control alligators deprived of food for 14 days was assayed for 18 amino acids. The results are shown in Table 1 along with those obtained by Harper, Hutchin & Kimmel (1952) for a group of fasting men. Curiously enough the composition of alligator blood is quite similar to that of man. In both species 50% or more of the total identified α-amino nitrogen was in the form of glycine, alanine, glutamine, valine and leucine. Of these five amino acids, all but valine proved to be precursors of urine ammonia. None of the other amino acids, which were present in lesser concentrations, contributed greatly to renal ammonia synthesis in either man or the alligator (Coulson & Hernandez, 1959).

When insulin was injected into alligators (Fig. 1) there was a slow decline from a value of about 4 m-moles of total amino acid/l. to a level approaching zero. It was not possible to determine whether there was any change in the relative ratio of these amino acids with respect to each other since their concentrations fell too low to be measured by our methods of analysis. In this experiment glucose concentrations were also measured and it is apparent that, apart from the phenomenon of an initial hyperglycaemia phase (Coulson & Hernandez, 1953), both glucose and amino acid concentrations decreased at about the same rate (Fig. 1). In this regard, at least, the effect of insulin on amino acids was as marked as it was on glucose.

L-*Amino acid tolerance in the alligator*

In the series of experiments illustrated in Fig. 2A and Fig. 2B the changes in the plasma amino acid levels were followed after the injection of amino acids into alligators with and without insulin. Where the amino acids disappeared very slowly from the plasma, the results of only the first 2 or 3 days appear in the figure owing to space limitations. Several methods of assessing the relative rates of utilization were tried and the most satisfactory index of metabolic activity was obtained by comparing the reciprocals of the respective areas under the curves with that of L-serine, the amino acid which disappeared at the fastest rate. If the area beneath the tolerance curve of L-serine is given an arbitrary value of 100%, then a tolerance curve for another amino acid which had twice the area of that of L-serine would be given a metabolic index value of 50%. Although this is a rough measure at best, it is still possible to say that L-serine was utilized many times as fast as L-methionine or L-glutamic acid.

D-*Amino acid tolerance in the alligator*

Although several of the D-amino acids were removed much more slowly than their respective natural isomers (Figs. 2A, 2B), about half were metabolized at the same rate and it was considered possible that the animals could have converted some of the D-forms into the L-isomers. To test this, eight of the slowly metabolized D-amino acids were injected into alligators and blood for analysis was removed 48 hr. later. The results of the microbiological assays of the L-amino acids in the plasma appear in Table 2. Injection of D-isoleucine and D-leucine did not lead to an increase, whereas each of the other six amino acids caused significant increases in the respective L-isomers. There is a problem of interpretation involved. Since an assay of all of the amino acid components was not attempted, it is possible that some of the other amino acids may have increased as well and that the magnitude of the small rise in the L-forms of valine, threonine and tryptophan may not have exceeded that of some of the others which were not determined. Although the results suggest isomerization, more experiments are needed for conclusive proof.

Cell permeability may also be an important factor in that in order to detect an increase in the L-isomer it must first be synthesized in the cell and then released into the plasma where it can be detected. The evidence does not permit the conclusion that the eight D-amino acids in Table 2 were selectively prevented from entering the cells since each of these compounds was distributed in about the same volume of water as its enantiomorph (Figs. 2A, 2B).

Effect of insulin on amino acid tolerance in the alligator

Although most of the amino acids of both isomers disappeared more rapidly when insulin was injected, there were a number of exceptions (Figs. 2A, 2B). It is difficult to formulate any logical rule

which would apply to the entire group. Some of the D-isomers were not as susceptible to the action of this hormone as their natural derivatives and in other cases insulin catalysed the removal of both isomers at the same rate. Valine was the only amino acid in which insulin affected the D-form significantly more than it did the L-form.

Insulin appeared to increase the toxicity of many of the amino acids whether the animals received glucose or not. This was a serious problem in the tryptophan experiments where the addition of insulin invariably killed the alligators within 48 hr. In the entire series of experiments thirty alligators died as a result of the amino acid injections and all

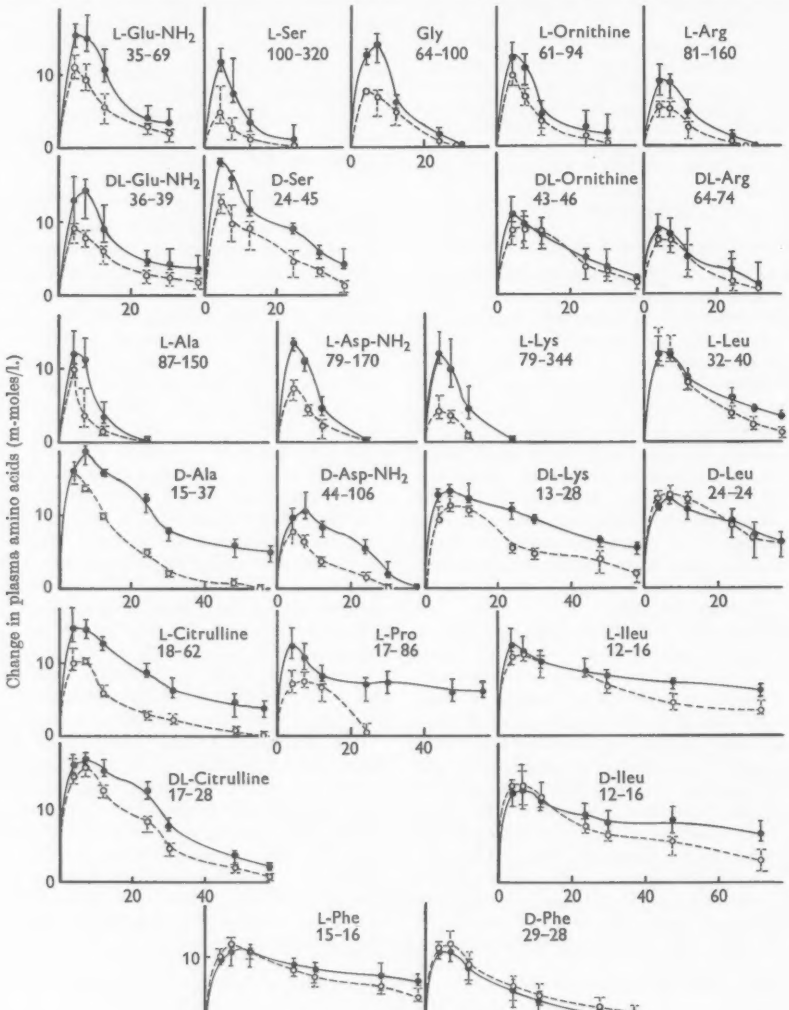

Fig. 2A. For legend see facing page.

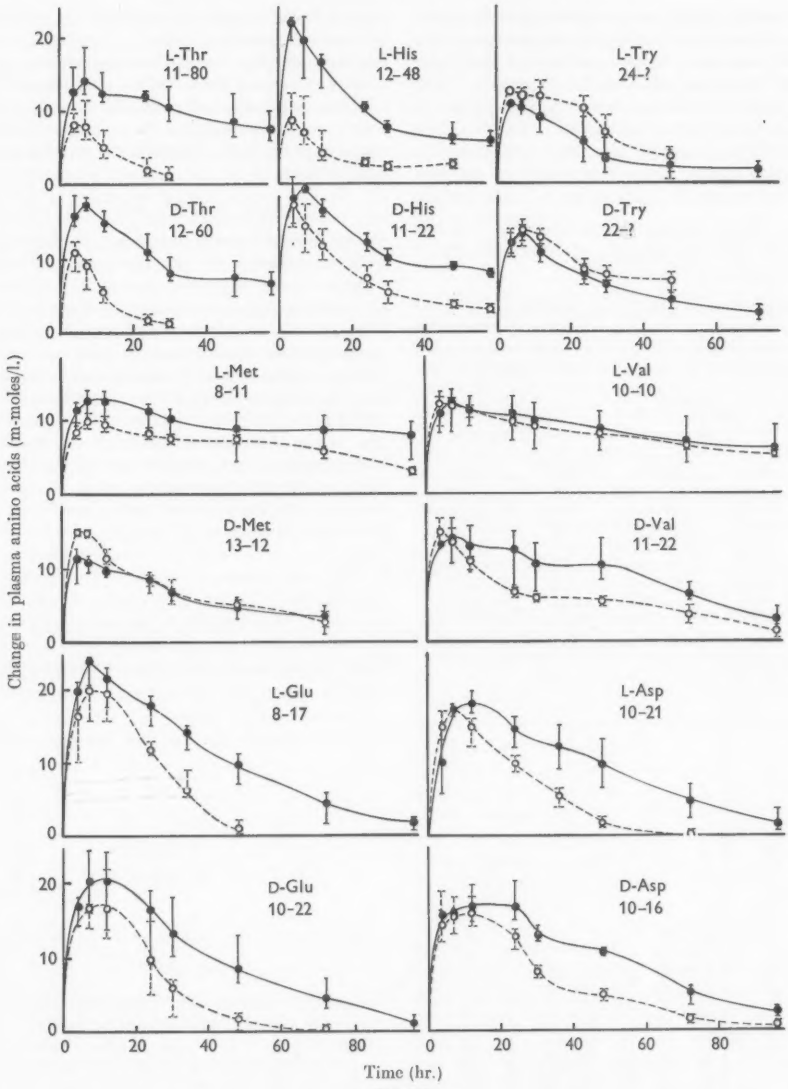

Fig. 2B

Figs. 2A and 2B. Amino acid tolerances in the alligator. Each amino acid (10 m-moles/kg. body wt.) was injected intraperitoneally into all animals. Four or more alligators were used in each of the groups. ●, Tolerance curves for the control groups; ○, tolerance curves for alligators treated with insulin. The perpendicular bars in the graph refer to the ranges of values encountered. As D-tryptophan was toxic and no alligator receiving insulin and D-tryptophan lived beyond 48 hr., the significance of these results is questionable. The first number in the upper right corner of each tolerance curve refers to the rate of utilization of each amino acid relative to that of L-serine, which was given an arbitrary value of 100% (see text). The second number refers to the rate found after injection of insulin. For example, L-glutamic acid without insulin was utilized at 8% of the rate of L-serine and insulin increased the rate to 17%. To conserve space, in some cases only, the values for the first 2–3 days were plotted. Several D-amino acids were unavailable and it was necessary to substitute the racemic mixtures.

but two were in the groups which received insulin. With the exception of D-serine no apparent specific difference was seen in the nature of the toxic symptoms occurring after different amino acids. The alligators vomited occasionally, gradually became torpid and died from causes which were not due to hypoglycaemia but were otherwise undetermined. The deaths were distributed at random among both D- and L-isomers of tryptophan, isoleucine, threonine, valine, citrulline, histidine, leucine, arginine, ornithine and serine. D-Serine always produced renal failure characterized by an initial anuria followed by death or by slow recovery over a period of months. None of the other amino acids were toxic enough to interfere with the amino acid tolerances.

Table 2. *Mean plasma concentration of* L-*amino acids* 48 *hr. after the injection of* 10 *m-moles/kg. of the corresponding* D-*isomers*

Three alligators were used in each experiment. The control values represent an average for 12 alligators deprived of food for two weeks. The plasma concentrations of the natural isomers were determined microbiologically.

D-Amino acid injected	L-Isomer (m-moles/l.) (Expt.) Mean	L-Isomer (m-moles/l.) (Expt.) Range	L-Isomer (m-moles/l.) (Controls) Mean	L-Isomer (m-moles/l.) (Controls) Range
Glu	4·51	(1·80–8·20)	0·07	(0·02–0·10)
Met	1·12	(0·71–1·92)	0·01	(Trace–0·02)
His	0·62	(0·48–0·79)	0·02	(0·01–0·03)
Val	0·40	(0·32–0·42)	0·13	(0·10–0·15)
Thr	0·20	(0·10–0·26)	0·07	(0·04–0·10)
Try	0·11	(0·07–0·19)	0·02	(0·01–0·02)
Ileu	0·06	(0·02–0·08)	0·04	(0·02–0·09)
Leu	Trace	(0·0 –0·02)*	0·11	(0·05–0·15)

* The D-isomer may have interfered with the growth of the assay organism.

Amino acid tolerance in the rat

The difficulties encountered in performing amino acid tolerances in the rat were many. Perhaps the most serious one was the variability of the results seen in replicate experiments. Fig. 3 is derived from the areas underneath the respective tolerance curves in the rat for some D- and L-amino acids. With L-serine receiving an arbitrary value of 100, the bar graphs compare the respective reciprocals of the areas derived from the amino acid tolerances and hence they are an index of the relative ease with which each amino acid was utilized by the rat. The rat metabolizes amino acids so much more rapidly than the alligator that it is difficult to compare their tolerances on the same scale. Some indication of the relative metabolic rates may be seen in Fig. 4 in which representative alligator and rat glutamic acid tolerance curves are compared. Since the area of the alligator curve is about 50 times that of the rat, the rat utilizes glutamic acid some 50 times as fast as the alligator. In general this ratio

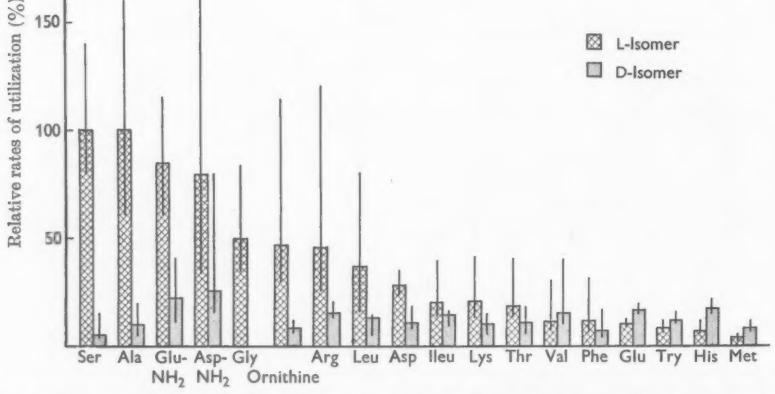

Fig. 3. Relative rates of utilization of L- and D-amino acids by the rat. The reciprocals of the areas of the rat tolerance curves are indicated by the heights of the rectangles. Since L-serine disappeared in the shortest time, and since this tolerance curve had the least area, it was given an arbitrary value of 100%. The perpendicular lines indicate the range. There were four or more rats in each experiment. Each rat received 10 m-moles of amino acid/kg. body wt.

prevailed for the other amino acids as well. The rapid removal of amino acids from rat plasma prevented an accurate assessment of their distribution in body fluids.

No satisfactory corrections for undoubted urinary losses of the D-isomers (Berg, 1953) in the rat were possible without the necessity for the complete isolation of these components in the urine. It was not possible to measure the unnatural isomers by microbiological assay, and ninhydrin reacts not only with the D-isomers but with the other α-amino metabolic derivatives as well. Fortunately urinary losses were not a problem in the alligator owing to the very low excretory rate of the kidney (Coulson & Hernandez, 1955). The inability of the alligator to excrete urine more concentrated than the plasma (280 m-osmoles/l.) prevented the excretion of amino acids in any quantity. For example, if the urine volume were 10 ml./kg./day and if half the solids in the urine were due to amino acids, the total excreted would be only a little over 1 m-mole/day or 10% of the amount injected. The true values were so much lower than this quantity that they were of no consequence in these experiments.

Conversion of histidine into glutamic acid

For a number of reasons experiments *in vivo* are unsuitable for determining the extent to which one compound is converted into another. For example, if a large amount of the injected glycine were converted into alanine, the increase in plasma alanine would necessarily be very small owing to the fact that it is also metabolized rapidly. However, if one amino acid were converted into a different amino acid which was utilized slowly, it might then be possible to detect a significant increase in the plasma concentration of the new derivative. Table 3 illustrates one such interconversion. It appears that both D- and L-histidine were converted into L-glutamic acid. In this instance at least, it was possible to show that a reaction well known in mammalian liver-slice studies also occurs in the intact alligator.

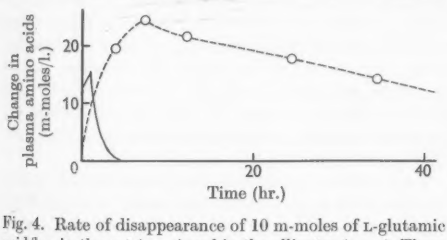

Fig. 4. Rate of disappearance of 10 m-moles of L-glutamic acid/kg. in the rat (——) and in the alligator (– – –). There were ten animals in each group. The sodium salt was injected in an iso-osmotic solution into the peritoneum.

DISCUSSION

When the height of the individual peaks in the amino acid tolerance curves are considered they fall into two general classes: those which reached a plasma concentration of about 20 m-moles/l. and those which reached a level of about 12 m-moles/l. It is logical to assume that for a brief time at least the class with the lower peaks must have been distributed in about twice the body-water volume of the class that reached the higher concentrations. An alligator of the size used in these experiments has an extracellular-fluid volume of about 400 ml./kg. body wt. and a total body-water volume of about 700 ml./kg. (Coulson & Hernandez, 1953). The injection of 10 m-moles of any non-utilizable substance would result in the attainment of a plasma concentration of about 14 m-moles/l. if it were distributed evenly in the body water, and of about 25 m-moles/l. if it were restricted to the extracellular spaces. Although absolute proof is lacking, the evidence would indicate that histidine, aspartic acid and glutamic acid are largely confined for a time to the extracellular fluids and that most of the others are more or less distributed evenly throughout the body water.

The rate at which any amino acid may be burned by an alligator is limited by the metabolic rate. On an average the complete oxidation of about 10 m-moles of an amino acid by a 1 kg. alligator would furnish all of the energy required for 1 day (Coulson & Hernandez, 1959). From the fact that several amino acids disappeared in about half a day, it must be assumed that either some form of storage occurred or that the amino acids were only partially degraded to carbon dioxide, water and uric acid. The slowly utilized compounds such as methionine

Table 3. *Conversion of* D- *and* L-*histidine into* L-*glutamic acid in the alligator*

Plasma was analysed 96 hr. after the injection of 10 m-moles of histidine/kg. body wt. There were four alligators in each experiment and four controls. All values are expressed as m-moles/l. of plasma.

Amino acid injected	Total amino acids (ninhydrin)		L-Glu (Microbiol.) (Expt.)		L-Glu (Microbiol.) (Controls)	
	Mean	Range	Mean	Range	Mean	Range
L-His	2·7	(2·0–3·3)	1·60	(1·38–1·83)	0·04	(0·02–0·10)
D-His	3·3	(1·8–5·1)	0·41	(0·29–0·65)	—	—

and glutamic acid could neither have functioned as efficient immediate energy sources nor as ready sources of raw material for protein synthesis in these experiments since the rate of their disposal was too low.

Some of the D-derivatives disappeared at a much slower rate than their respective natural isomers. The decreased ability to utilize the unnatural forms must have been associated with a decreased ability of the alligator to deaminate them. If the proper D-amino acid oxidases were present, either they were not in contact with the substrate, or they were present in too low a concentration to be effective. The existence of considerable concentrations of seemingly useless D-amino acid oxidases in the liver and kidney of several species of animals has long been a curiosity. These studies *in vivo* have not established the existence of such functional enzymes in the intact alligator.

The rate at which any amino acid of either configuration disappears from alligator plasma must be associated in some way with the rate at which it is used for protein synthesis, the rate at which it can be deaminated, or both. Those amino acids that remain in the extracellular fluid for days could not have entered rapidly into any deamination or transamination reactions. If the amino group had been removed, microbiological assay would have indicated the disappearance of the compound injected. That this did not occur was indicated by the fact that the ninhydrin-reacting substance found in the plasma 4 days after the injection of L-methionine was identified as unchanged methionine by microbiological assay.

There was a striking difference between the rate of removal of the essential amino acids and that of the non-essential amino acids. If one considers that arginine is not essential, then it can be said that of the essential compounds only lysine was removed rapidly by the alligator and only leucine by the rat. Conversely all of the dispensable amino acids were utilized very quickly by both species with the exception of glutamic acid and aspartic acid, which were metabolized with difficulty. This difference between the two types of amino acids probably illustrates a physiological adaptation in which the dispensable ones are used as a ready source of energy in contrast with the indispensable ones which are reserved for protein synthesis.

The relative rates of metabolism of various amino acids in the intact rat were in general similar to those in the alligator, which suggests that the metabolic pathways involved are similar for both species. It is generally assumed (although almost never stated) that transaminases and L-amino acid oxidases are ordinarily utilized *in vivo* in the catabolism of the various amino acids subject to their actions. When the activity of rat-kidney L-amino acid oxidase was tested by the successive addition of different amino acids *in vitro*, oxidative deamination occurred most rapidly with L-leucine as the substrate (Blanchard, Green, Nocito & Ratner, 1944). The decreasing order of activity was leucine, methionine, proline, isoleucine, tryptophan, valine, alanine etc. We found no correlation between the results of these studies *in vitro* and the rate at which the amino acids disappeared from the plasma of either the intact rat or the alligator. The compounds deaminated most readily *in vitro* usually had the lowest metabolic rate *in vivo*.

If it is assumed that the use of L-amino oxidases represented only a minor pathway of metabolism, and that the mechanism of transamination is the ordinary means by which these compounds are degraded, new theoretical problems arise. If most amino acids transfer their amino groups to such acceptors as α-oxoglutarate and oxaloacetate, the net result would be the formation of glutamate and aspartate. Since neither of these substances is metabolized rapidly *in vivo*, this would represent a rate-limiting step. It is difficult to conceive how large amounts of many of the non-essential amino acids could disappear in such a short time by such a route.

In general there appears to be very little correlation between the structure of an amino acid and the amount of influence insulin has upon its rate of removal. The absence of any effect of insulin on valine, leucine and isoleucine may indicate that this hormone is not active in accelerating the metabolism of certain branched-chain compounds. It is very difficult to visualize the effect of insulin in terms of the activation of any single enzyme system and it is also difficult to understand its effects solely in terms of increased cell permeability. For example, threonine is distributed in body water and glutamic acid is restricted to the extracellular fluid. It might be expected that insulin would drive glutamic acid across the cell barrier and hence that its effects would be much greater than on threonine, which is already available to the cell. The reverse was true.

Sugars which are metabolized more rapidly under the influence of insulin have the same configuration with respect to the first three carbon atoms (Levine & Goldstein, 1955). This and other similar observations inclined many observers toward the view that the classical 'lock and key' principle is involved. According to many, the observed effects of insulin on fats and proteins are most easily explained on the basis of their direct effects on carbohydrate metabolism. Although such theories have the advantage of simplicity it is difficult to apply them to explain the observed effects of insulin on all of the different amino acids of both configurations. It is possible that insulin affects directly more types of compounds than is commonly believed.

SUMMARY

1. By taking advantage of the low metabolic rate of the alligator, it was possible to study the effect of insulin on the rate at which each of a large group of amino acids was utilized *in vivo*.

2. 'Amino acid tolerances' of both isomers of amino acids were determined in the alligator and in the rat by the injection of 10 m-moles/kg. followed by analyses of plasma amino acids at frequent intervals. The rat metabolized amino acids from 50 to 100 times as fast as the alligator. In general, the non-essential amino acids were utilized much more rapidly than the essential ones by both species.

3. Each amino acid had its characteristic tolerance curve.

4. In the alligator, insulin increased the rate of metabolism of most of the exogenous amino acids of both isomers.

5. Insulin appears to enhance the metabolism of compounds which differ greatly in chemical structure.

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The Effect of Testicular Hyaluronidase on the Hyaluronic Acid Complexes of Human Synovial Fluid

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It has been shown that the electrophoretic mobility of the human synovial-fluid proteins is affected by the viscosity of the solution (Pigman, Patton & Platt, 1957). This effect was interpreted as a formation of a complex of the viscous component of synovial fluid, hyaluronic acid, with the proteins. Evidence for this formation has been given by a number of investigators (Ropes, Robertson, Rossmeisl, Peabody & Bauer, 1947; Ogston & Stanier, 1950, 1952; Curtain, 1955; Blumberg & Ogston, 1957; Pigman *et al.* 1957; Hamerman & Sandson, 1959; Pigman, Gramling, Platt & Holley, 1959). A component moving with a mobility between those for hyaluronic acid and albumin was found in many normal and post-mortem human synovial fluids (Platt, Pigman, Holley & Patton, 1956; Pigman *et al.* 1957). This component has been named the π-peak (Platt *et al.* 1956) and was believed to be a stable complex between hyaluronic acid and protein (Platt, 1957). The present investigation was undertaken to ascertain whether the π-peak is a non-dissociable complex between hyaluronic acid and protein, and whether the increase in the electrophoretic mobility of the synovial proteins is solely the result of an increase in viscosity.

MATERIALS AND METHODS

Human synovial fluids. The human synovial fluids were aspirated *post mortem* from the knee joint at hospital autopsy within a few hours after the death of the patient. All fluids were obtained from individuals with no history of joint symptoms or arthritic disease. When the amount of fluid was deemed insufficient for electrophoretic analysis, two or more fluids were pooled. The samples were placed in sterile containers and kept under refrigeration until they were analysed.

Hyaluronidase treatment. Two types of enzymic treatment were used: for mild treatment with hyaluronidase, the synovial fluids were treated with 2 vol. of buffer (pH 8·6, see below) and incubated at 30° for 2 hr. after the addition of 1 T.R.U. (turbidity reducing unit) of testicular hyaluronidase/ml. of synovial fluid. During this period of time, the solution was shaken vigorously for 1 min. at 15 min. intervals. The sample was then dialysed against 500 ml. of buffer for 48 hr. at 2–4°. For more drastic treatment with hyaluronidase, 3 T.R.U. of hyaluronidase/ml. of synovial fluid was added to the undiluted synovial fluid. The solution was incubated at 37° for 2 hr. and shaken vigorously at 15 min. intervals. At the end of the incubation period, the fluid was diluted with 2 vol. of buffer and dialysed as described above. The buffer used was sodium diethyl barbiturate (pH 8·6, *I* 0·1).

The testicular hyaluronidase (1250 T.R.U./mg.) was obtained from Wyeth Laboratories, Philadelphia, Pa., U.S.A.

Electrophoretic analysis. The fluids after dilution and dialysis were analysed by using a Perkin–Elmer Tiselius-type electrophoresis apparatus with the 2 ml. cell at 0·5°, with a constant current of 6 mA. The time of the runs ranged from 6000 to 8200 sec. The electrophoretic mobilities were calculated from the ascending patterns for reasons previously described (Platt *et al.* 1956; Pigman *et al.* 1957).

Viscometry. Viscosity measurements were made at 30° with two types of viscometers, a pipette-type viscometer (Pigman *et al.* 1957) and Cannon–Manning capillary semi-microviscometers (Cannon Instrument Co., State College, Box 812, Pa., U.S.A.).

RESULTS

The results of the electrophoretic analyses of human synovial fluid before treatment with testicular hyaluronidase are shown in Table 1. Of the ten fluids analysed, all contained hyaluronic acid, albumin and α_1- and β-globulin. Six of the samples had a detectable α_2-globulin component, and four a γ-globulin component. A π-peak was present in the patterns for four fluids. Hyaluronic acid always moved as a sharp peak. The viscosity range of the fluids was 6·3–30·8 cP. All protein components with the exception of γ-globulin exhibited a mobility which increased with the viscosity of the solution.

After treatment with hyaluronidase, the viscosities of the fluids dropped markedly, as shown in Table 2. The viscosity ranged from 0·9 to 4·7 cP. All the samples contained hyaluronic acid, albumin and α_1- and β-globulin. In eight of the fluids the α_2-globulin peak could be detected, and in seven a γ-globulin component was present; in some of these fluids these components were demonstrated only after the enzyme treatment. The π-peak which was found in four of the fluids before hyaluronidase treatment could not be detected in any subsequent to the treatment. The profile of the hyaluronic acid peak appeared to be only slightly affected by enzymic treatment, which produced a broadening of the base of the peak.

The mobility of the hyaluronic acid was essentially unchanged by treatment with hyaluronidase. The mobilities of the synovial proteins decreased markedly after the action of hyaluronidase. Although the greatest viscosity of the treated fluids was five times as great as that of the least viscous fluids, there was little variation in the mobilities of the individual components in the enzyme-treated fluids. The mobilities of the protein

Table 1. *Electrophoretic mobilities of human synovial-fluid components*

The values in columns 2–8 are given as $-10^5 \times$ electrophoretic mobility (cm.^{2} v^{-1} sec.$^{-1}$). — Indicates not found.

Fluid	Hyaluronic acid	π	Albumin	Globulins				Viscosity (centipoises)
				α_1	α_2	β	γ	
A	9·3	—	7·3	6·5	5·2	3·7	1·5	6·3
B	9·1	—	7·4	6·6	—	4·0	1·8	9·0
C	9·5	—	7·6	6·2	—	4·7	—	14·5
D	9·5	8·0	7·7	7·1	—	4·6	—	14·5
E	9·2	8·2	7·8	7·2	5·5	4·7	—	16·7
F	9·2	8·4	7·9	7·2	5·7	5·0	2·1	20·3
G	9·0	—	8·1	7·1	5·8	5·1	—	23·7
H	9·1	8·6	8·2	7·2	—	5·3	—	24·6
I	9·0	—	8·4	7·0	6·0	5·6	—	27·2
J	9·4	—	8·8	7·3	6·4	5·9	1·8	30·8
Average	9·3	8·3	7·9	7·0	5·8	4·9	1·8	—
Range	9·0–9·5	8·0–8·6	7·3–8·8	6·2–7·3	5·2–6·4	3·7–5·9	1·5–2·1	—
Serum*	—	—	6·4	5·5	4·5	3·5	1·4	0·9

* Pigman, Patton & Platt (1957).

Table 2. *Electrophoretic mobilities of human synovial-fluid components after treatment with testicular hyaluronidase*

The values in columns 2–7 are given as $-10^5 \times$ electrophoretic mobility (cm.^{2} v^{-1} sec.$^{-1}$). The first letter denoting the fluid analysed corresponds to the letter in Table 1. (The final letter H refers to treatment with hyaluronidase.)

Fluid	Hyaluronic acid	Albumin	Globulins				Viscosity (centipoises)
			α_1	α_2	β	γ	
AH	8·9	6·4	5·4	4·7	3·4	1·8	0·9
BH	9·1	6·4	5·5	4·5	3·4	1·7	1·3
CH	9·1	6·6	5·6	—	3·7	—	1·6
DH	9·0	6·7	5·7	5·0	3·8	1·4	4·4
EH	9·3	6·6	5·5	4·6	3·7	1·6	1·2
FH	9·1	6·5	5·6	4·7	3·7	1·7	4·6
GH	9·1	6·6	5·6	4·7	3·6	1·4	4·2
HH	9·3	6·5	5·4	4·6	3·5	—	1·8
IH	9·1	6·4	5·6	—	3·8	—	4·7
JH	9·0	6·6	5·6	4·7	3·7	1·7	4·7
Average	9·1	6·5	5·6	4·7	3·6	1·6	—
Range	8·9–9·3	6·4–6·7	5·4–5·7	4·5–5·0	3·4–3·8	1·4–1·8	—
Serum*	—	6·4	5·5	4·5	3·5	1·4	0·9

* Pigman *et al.* (1957).

components for the treated fluids were similar to that obtained for blood serum, which has a viscosity of 0·9–1·0 cP under similar conditions. γ-Globulin appeared to be the least affected by treatment with hyaluronidase.

The disturbances often noted in the descending limb when the untreated samples were analysed were not present in the hyaluronidase-treated fluids. The electrophoretic patterns appeared to be 'sharper' in the hyaluronidase-treated fluids except for the hyaluronic acid component. A slight decrease seemed evident in the albumin/globulin ratios after treatment with hyaluronidase, but this decrease did not appear to be significant.

DISCUSSION

It was postulated that the increase in mobilities of the synovial-fluid proteins that occurs with an increase in the viscosity of that fluid may arise from complex-formation between hyaluronic acid and the synovial proteins (Pigman *et al.* 1957). Curtain (1955) showed that the hyaluronic acid complex, isolated by ultrafiltration from synovial fluid, on electrophoresis contained, in addition to hyaluronic acid, proteins which behaved similarly to the major serum components.

The loss of the π-component on treatment with hyaluronidase suggests that it consists of hyaluronic acid loosely associated with proteins. In other work (Pigman, Gramling & Holley, 1960), π-components have been formed in mixtures of highly polymerized hyaluronic acid and serum albumin under the conditions of the present work. Katchalsky (1954) has indicated that the electrostatic fields of macromolecules are sufficiently powerful to permit the cross-linking of macromolecules by electrovalent bonds, despite the dissociative action of water resulting from its high dielectric constant. This concept suggests that the ionized carboxyl groups of hyaluronic acid can react directly with the cationic amino groups of the proteins. Even in the virtual absence of charged groups in one polymer, the interaction between a polyelectrolyte and a 'neutral' polymer resulted in the formation of a new component in the mixture (Platt, 1960). The complex formed between sodium carboxymethylcellulose and the 'neutral' polymers, methylcellulose and polyacrylamide, was stable in an electrical field.

The degree of polymerization of hyaluronic acid seems to be important in complex-formation. The apparent effect of viscosity on the electrophoretic mobilities of the proteins suggests a direct correlation between mobility and viscosity. However, the viscosity of some of the fluids treated with hyaluronidase was similar to that of untreated fluids in the earlier study (Pigman *et al.* 1957), but, in the hyaluronidase-treated fluids, no effect of viscosity on mobility could be detected. This result indicates that degradation of the hyaluronic acid molecule resulted in a breakdown of the hyaluronic acid complex and that partially depolymerized hyaluronic acid will not form the complex. That high-molecular-weight hyaluronic acid is required for the formation of the π-complex was also demonstrated by the earlier failure to produce a π-peak by the addition of low-molecular-weight hyaluronic acid to synovial fluid and blood serum (Platt, 1957).

In contrast with the electrophoretic work, testicular hyaluronidase has been shown to exert little

or no effect on the sedimentation behaviour of synovial fluid (Platt, Pigman & Holley, 1959) and the hyaluronic acid complex (Fessler, 1960).

SUMMARY

1. Human synovial fluid was analysed electrophoretically before and after treatment with testicular hyaluronidase.

2. After hyaluronidase treatment, the electrophoretic mobilities of the synovial proteins decreased markedly and were similar to the values obtained for blood serum proteins.

3. The π-component, a complex of hyaluronic acid and albumin, could not be detected after hyaluronidase treatment.

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The Control of some Oxidative Pathways in Guinea-Pig Mammary-Gland Mitochondria

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The control of metabolic pathways can occur on three different time scales. First, the steady-state levels of metabolite concentrations can be changed within seconds by the activation or inhibition of enzymes due to the introduction of metabolites, hormones or other modifiers. These rapid changes can involve the release of preformed enzymes but are not likely to involve synthesis of enzymes. The second time scale covers a long range from minutes to days, which are the periods required for adaptive enzyme formation. In this paper we are concerned with a third and very much longer time scale required for tissue to adapt itself to a special purpose during the evolution of the organism. For the biosynthesis of proteins and fatty acids, the mammary gland is the specialized organ *par excellence*. Terner (1951) has already shown that the tricarboxylic acid cycle functions in mammary tissue. The present work was undertaken as part of a study of the biochemistry of the mammary gland. We are also interested in examining mitochondria, from specialized tissue, for quantitative differences in enzyme activities of steps of the citric acid cycle, and of steps involved in the supply and utilization respectively of substrates and products of the cycle. A study of a wide range of oxidative and other metabolic reactions can be shown to reflect the predominantly anabolic activity of the mammary gland.

Many different criteria have been formulated to define intact mitochondria. We have relied upon the evaluation of the P/O ratio (phosphorylation of adenosine diphosphate to adenosine triphosphate/oxygen uptake) in the presence of different substrates and of respiratory control (respiration in the presence and absence of the phosphate acceptor, adenosine diphosphate) (Lardy, 1956) as a measure of the biochemical integrity of our mitochondrial preparations. By far the largest number of investigations on mitochondria has been carried out with preparations from liver. From this source good preparations can be obtained with relative ease by

a variety of methods. The mammary gland, like some other specialized tissues, has, however, presented greater difficulties and the isolation of intact phosphorylating mitochondria from such sources has only been reported recently when Ciaccio & Nelson (1958) and Jones & Gutfreund (1959) were successful with such preparations. Guinea-pig mammary tissue is still the only one which can be homogenized sufficiently gently so that mitochondria are not damaged. The choice of glands from guinea pigs together with some care in the removal of milk constituents, especially of calcium, are the two factors mainly responsible for the success in obtaining satisfactory mammary-gland mitochondria. As will be seen, the yields and reproducibility of mammary-gland mitochondria are still not up to the level now universally achieved from liver preparations, but the results of our studies show that the mitochondria of a tissue with such specialized synthetic activity have features which make them a most useful system for the study of the enzymic control of its anabolic processes.

EXPERIMENTAL

Preparation of mitochondria

Liver mitochondria were prepared from young male guinea pigs by the procedure of Schneider (1948). Mammary-gland mitochondria were prepared from guinea pigs 2–6 days after parturition. The animals were stunned and bled by cutting their throats, and the glands excised. All subsequent operations were carried out at 0–5°. The glands were passed through a Latapie mincer into approximately twice their weight of cold medium [0·25 M-sucrose, 5 mM-ethylenediaminetetra-acetate, adjusted to pH 7·4 with KOH). The mince was squeezed dry in a muslin bag and homogenized with fresh medium in a Potter homogenizer with a nylon pestle to give an approximately 20% homogenate (g. of fresh gland tissue per volume of homogenate). The homogenate was first centrifuged for 5 min. at 600*g* to remove debris and then for 15 min. at 12 000*g* to deposit the mitochondria. The mitochondria were resuspended in half the original volume of medium and again centrifuged for 15 min. at 12 000*g*. All centrifugings were carried out in a refrigerated Spinco preparative ultracentrifuge. The final mitochondrial pellet was again resuspended in the above medium and stored at 2–4°; experiments were carried out within 3 hr. of completing the preparation. Kjeldahl N determinations were carried out in most cases on these mitochondrial suspensions, the N content of the suspension medium was subtracted from the blank. In some experiments only relative rates are recorded; this was done when only small samples were available and we did not reserve any material for N estimations.

Determination of oxygen uptake and phosphorylation

Two methods were used for the measurement of the rate of O_2 uptake by mitochondria, first the conventional Warburg technique and secondly the polarographic technique. Experiments were carried out at 25° or 30°.

The polarographic apparatus consisted of a pair of electrodes, one a gold wire, the other a silver wire, vibrating at 50 cyc.; they were insulated by polythene tubing except at the tips. The gold electrode was put at a negative potential of 0·8 v with respect to the silver electrode and the flow of current was recorded on a polarograph (Evershed and Vignoles Ltd., London, W. 4). Each experiment is self-calibrating on the basis that the O_2 concentration is linearly related to the polarographic current and that the start of the experiment gives the current for a 250 μM-solution of O_2, whereas the final anaerobic solution gives the residual current at zero O_2 concentration.

The electrodes are immersed in 5 ml. cuvettes containing 3 ml. of reaction mixture; the cuvette is immersed in a thermostat. The incubation medium for polarographic measurements (medium A) of the rate of O_2 uptake consisted of a solution: 8 mM-KH_2PO_4, 0·02 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), 8 mM-$MgSO_4$, 0·1 M-sucrose (pH 7·4); substrates, inhibitors and ADP were added to give the concentrations stated for each experiment; mitochondrial suspensions (0·5 ml.) were added to start the experiments. In experiments proceeding at such a rate that all the O_2 of the solution was used up in about 10 min., no correction was required for diffusion or stirring in of O_2.

For some of the measurements of O_2 uptake and of oxidative phosphorylation the Warburg technique was used with the following medium (medium B): 8 mM-KH_2PO_4, 0·02 M-tris, 8 mM-$MgSO_4$, 8 mM-glucose, 4 mM-adenosine triphosphate (ATP), 2 mM-substrate, 0·5 mg. of hexokinase (Pabst Laboratories, Milwaukee, Wisc., U.S.A.) (pH 7·4). Consumption of O_2 during the equilibration period of 10 min. was assumed to be equal to that during the first equivalent measured period. Phosphorylation was measured by trapping phosphate as glucose 6-phosphate formed from the generated ATP and glucose in the presence of hexokinase; the disappearance of inorganic phosphate from the medium was determined by application of the method of Fiske & Subbarow (1925) to a sample of the trichloroacetic acid filtrate. The reaction was terminated by tipping 0·3 ml. of 40% (w/v) trichloroacetic acid from the side arm of the Warburg flask.

All the substrates as well as adenosine diphosphate (ADP) and ATP were made up as potassium salts, pH 7·4.

A number of experiments were carried out to test the agreement between measurements of O_2 consumption as recorded by the polarographic technique and by the Warburg method at 25°. Data obtained from succinate oxidation by heart-muscle preparation (Keilin & Hartree, 1940) and from the respiration of liver and mammary-gland mitochondria in the presence of α-oxoglutarate showed that the two methods gave results which agreed within 5%.

RESULTS

Yields and integrity of mammary-gland mitochondria

A portion (60 ml.) of a 20% (w/v) mammary-gland homogenate yielded mitochondria and debris, which were made up to volumes of 9 and 13·5 ml. respectively. From Warburg experiments with samples of the two fractions it was calculated that at 30° the whole of the debris fraction oxidized

140 μmoles of citrate/hr. and 232 μmoles of α-oxoglutarate/hr. The oxygen consumption was 452 μg.-atoms/hr. and 712 μg.atoms/hr. in the presence of added citrate and α-oxoglutarate respectively. The whole of the mitochondrial fraction with added citrate oxidized 104 μmoles of citrate/hr., with an oxygen uptake of 304 μg.atoms/hr.; with added α-oxoglutarate it oxidized 164 μmoles of α-oxoglutarate and the oxygen consumption was 392 μg.atoms/hr. Re-homogenization of the debris yielded more respiratory activity in a second mitochondrial fraction, but this was not carried out as a routine. In a comparison with the yield of guinea-pig-liver mitochondria, it was found that the mitochondria obtained from a gram of liver mince had an oxygen uptake of 96 μg.atoms/hr. with added α-oxoglutarate, whereas mitochondria from mammary gland consumed 21 μg.atoms of oxygen/hr. in the presence of α-oxoglutarate. Re-extraction of the debris from mammary-gland homogenate yielded an equal amount of activity.

It has been stated that respiratory control and P/O ratios, as defined above, have been taken as criteria for the integrity of our mitochondrial preparations. It would be useless to quote mean values for these ratios since the mammary-gland preparations show considerable variability. We suggest that since we have used only such preparations for critical experiments which gave respiratory control of 3 or more it is sufficient to say that values between 3 and 6 can be, but are not always, obtained. Figs. 1*A* and *B* show the response of good preparations of mammary gland and liver mitochondria to the addition of ADP. Similarly, it is justifiable to quote the highest

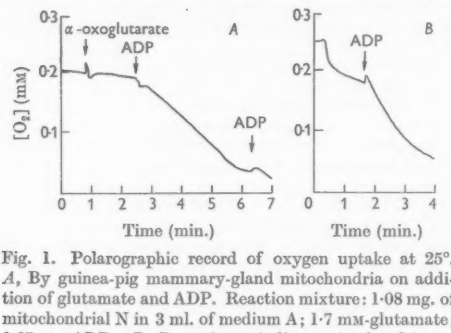

Fig. 1. Polarographic record of oxygen uptake at 25°. *A*, By guinea-pig mammary-gland mitochondria on addition of glutamate and ADP. Reaction mixture: 1·08 mg. of mitochondrial N in 3 ml. of medium A; 1·7 mM-glutamate; 0·67 mM-ADP. *B*, By guinea-pig-liver mitochondria on addition of α-oxoglutarate and ADP. Reaction mixture: 1·2 mg. of mitochondrial N in 3 ml. of medium A; 1·7 mM-α-oxoglutarate; 0·67 mM-ADP.

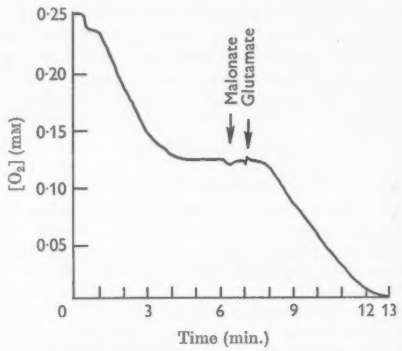

Fig. 2. Polarographic record of oxygen uptake at 25° by guinea-pig mammary-gland mitochondria in the presence of malate and ADP and on subsequent addition of malonate and glutamate. Reaction mixture: 0·72 mg. of mitochondrial N in 3 ml. of medium A; 1·7 mM-malate; 3·3 mM-malonate; 1·7 mM-glutamate; 1 mM-ADP.

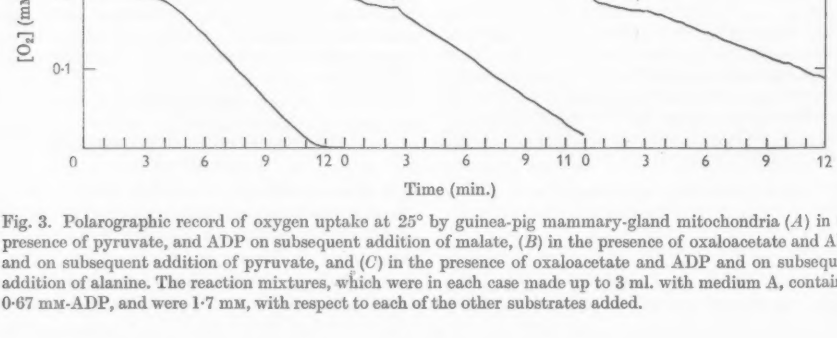

Fig. 3. Polarographic record of oxygen uptake at 25° by guinea-pig mammary-gland mitochondria (*A*) in the presence of pyruvate, and ADP on subsequent addition of malate, (*B*) in the presence of oxaloacetate and ADP and on subsequent addition of pyruvate, and (*C*) in the presence of oxaloacetate and ADP and on subsequent addition of alanine. The reaction mixtures, which were in each case made up to 3 ml. with medium A, contained 0·67 mM-ADP, and were 1·7 mM, with respect to each of the other substrates added.

values obtainable for P/O ratios from our preparations of mammary-gland mitochondria: on addition of succinate, 1·4; on addition of α-oxoglutarate in the presence of malonate, 3·8; on addition of citrate, 2·5.

Rate of oxidation of citric acid cycle intermediates

The q_{O_2} (μg.atoms/mg. of mitochondrial nitrogen) values at 25° for both liver and mammary-gland mitochondria in the presence of citrate, α-oxoglutarate, succinate and malate were all indistinguishable and within the range 13–15. In the presence of oxaloacetate, liver mitochondria gave a q_{O_2} of 9, whereas mammary-gland mitochondria produced no significant oxygen uptake (see Fig. 3*B*). Another important difference between the mitochondria from the two tissues lies in the failure of mammary-gland mitochondria to oxidize malate after the period of equilibration in Warburg experiments. Fig. 2 shows the polarographic record of the oxygen uptake by mammary-gland mitochondria in the presence of malate. When the oxygen concentration of the solution is lowered from 250 to 115 μM respiration stops. On the assumption that all the oxygen has been used for the conversion of malate into oxaloacetate one can calculate that the respective concentrations are 1·73 and 0·27 mM when the oxidation of malate stops. In the Warburg method malate oxidation will cease during equilibration, before the first reading is taken. No significant uptake of oxygen is observed by either method in the presence of oxaloacetate. Fig. 3*A* and *B* give the records of the relative rates of oxygen uptake by mammary-gland mitochondria in the presence of pyruvate alone, oxaloacetate alone, pyruvate plus oxaloacetate and pyruvate plus malate and illustrate that both malate and oxaloacetate oxidation are stimulated by the addition of pyruvate. Fig. 3*C* illustrates the stimulation of oxaloacetate oxidation by alanine, and Fig. 2 that of malate by glutamate in the presence of malonate, which inhibits glutamate oxidation (Fig. 5*B*) in mammary-gland mitochondria.

The steady-state rate of respiration in the presence of malate and pyruvate is 1·5 times as fast as in the presence of oxaloacetate and pyruvate. This illustrates a difference in rate control of the citric acid cycle to be discussed below together with the usefulness of mitochondria with a block in oxaloacetate oxidation in the absence of added pyruvate as a tool for the study of a variety of phenomena.

Metabolism of glutamate

An assay method was developed which allows one to determine the rate of reduction of diphosphopyridine nucleotide (DPN) by various dehydrogenases in the presence of negligible amounts of reduced diphosphopyridine nucleotide (DPNH) (H. Gutfreund & E. A. Jones, in preparation); this is based on the procedure suggested by Ells (1959) and involves the reduction of 2:6-dichlorophenol-indophenol by DPNH in the presence of catalytic concentrations of phenazine methosulphate. We used these conditions for the determination of the activity of glutamic dehydrogenase of mitochondria which had been stored frozen. These preparations did not oxidize α-oxoglutarate and the rate of appearance of DPNH in the presence of glutamate (10 mM), DPN (0·1 mM) and phenazine methosulphate (0·05 mM) could, therefore, be attributed entirely to the reaction:

$$\text{Glutamate} + \text{DPN}^+ + \text{H}_2\text{O} \rightarrow \alpha\text{-oxoglutarate} + \text{NH}_3 + \text{DPNH} + \text{H}^+$$

Preliminary studies indicate that the glutamic dehydrogenase of guinea-pig-liver mitochondria can under these conditions reduce 0·1 m-mole of DPN/hr./mg. of N at 20° in the presence of 10 mM-glutamate. In contrast with this very high activity of glutamic dehydrogenase in liver mitochondria, no activity could be detected in mammary-gland mitochondria.

Two experiments were carried out with guinea-pig-liver mitochondria to gain some information about the inhibition of the glutamic-dehydrogenase reaction by the addition of α-oxoglutarate under the assay conditions described above. With 9 mM-glutamate and 1 mM-α-oxoglutarate the rate of reduction of DPN was lowered by a factor of 3 over the rate obtained in the presence of 10 mM-glutamate alone; with substrate and product present at equal concentrations (5 mM each) no reduction of DPN could be detected.

Figs. 4*A* and *B* and 5*A* and *B* illustrate the rate of oxygen uptake by liver and mammary-gland mitochondria in the presence of glutamate or α-oxoglutarate and the inhibition of these reactions by malonate. The q_{O_2} at 25° for the two experiments in the presence of glutamate alone is 13 for liver and 12 for mammary gland. The apparent rate of glutamate oxidation for the two different mitochondria is remarkably similar, considering the great difference observed for the activity of glutamic dehydrogenase.

Fig. 5*B* illustrates the inhibition of glutamate oxidation by malonate and the revival of respiration on addition of malate.

DISCUSSION

The experiments described in this paper were designed to investigate a number of metabolic properties of guinea-pig mammary-gland mitochondria and to compare these with properties of

liver mitochondria. Such differences as have been established can be usefully analysed from the point of view of their contribution to the control of the synthetic activity of such a very highly specialized tissue as the mammary gland.

First of all it has to be established that mammary-gland mitochondria can be prepared which have a sufficient number of critical properties in common with liver mitochondria so that one can be satisfied about their integrity; only then is it justifiable to interpret any differences as due to different properties of specialized intact mitochondria. It is for instance of fundamental importance that experiments on the control of the citric acid cycle by substrates and products are carried out with mitochondria which do not only maintain their *in vivo* complement of citric acid cycle enzymes during isolation but which also preserve the close coupling between the dehydrogenases and electron transport to oxygen and phosphorylation of ADP to ATP. Any disruption of these naturally interlinked processes would change the energy balance and could give misleading results by an alteration of the steady-state control of dehydrogenase reactions.

Respiratory control in living tissue may be defined as the ability to accelerate its rate of respiration during activity and to slow down its rate of respiration at rest when the expended energy has been restored. For isolated mitochondria respiratory control can be studied as illustrated in Fig. 1. The mitochondria show a clear response to the addition of a phosphate acceptor (ADP).

The fact that the q_{O_2} values for liver and mammary-gland mitochondria were approximately the same for most substrates of the citric acid cycle does not mean that the concentrations of the individual enzymes are necessarily the same in the two tissues. This point will have to be investigated separately. It does mean, however, that the rate of control of overall coupled dehydrogenation and electron transport for the DPN-linked steps of the cycle is the same for the two tissues. The two mitochondrial enzyme reactions which have been found to be markedly different in liver and mammary gland (glutamic dehydrogenase and oxaloacetate decarboxylase) are concerned with the supply or disposal of citric acid cycle intermediates. This may well be a more general phenomenon since no marked differences in enzyme activities of the citric acid cycle have been reported, whereas the supply lines to the cycle have been shown to exhibit marked variations; Zebe (1960), for instance, showed that in locust flight-muscle the enzyme reaction involved in the supply of citric acid cycle intermediates from fatty acids can be between 10 and 100 times as fast as in tissues from other animals. The rate of respiration of

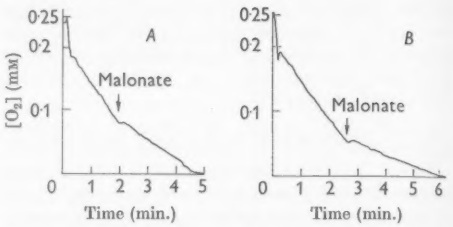

Fig. 4. Polarographic record of oxygen uptake at 25° by guinea-pig-liver mitochondria (*A*) in the presence of α-oxoglutarate and on subsequent addition of malonate, and (*B*) in the presence of glutamate and on subsequent addition of malonate. Each reaction mixture contained, in 3 ml. of medium A, 1·82 mg. of mitochondrial N, and was 1·3 mM with respect to ADP; the other substrates were added to give final concentrations of 1·7 mM.

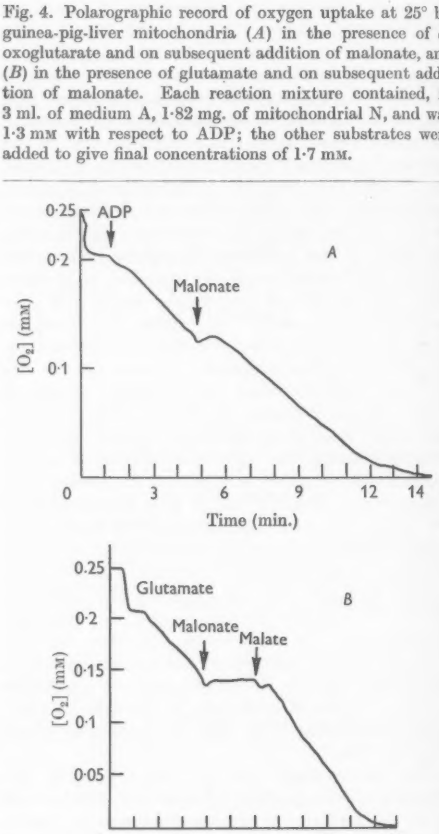

Fig. 5. Polarographic record of the rate of oxygen uptake at 25° by guinea-pig mammary-gland mitochondria (*A*) in the presence of α-oxoglutarate and on subsequent addition of malonate, (*B*) in the presence of glutamate and on subsequent successive addition of malonate and malate. Each reaction mixture contained, in 3 ml. of medium A, 0·72 mg. of mitochondrial N, and was 1 mM with respect to ADP; the substrates were added to give a final concentration of 1·7 mM and malonate to give a final concentration of 3·3 mM.

mammary-gland mitochondria in the presence of malate and pyruvate or of citrate is 1·5 times as fast as in the presence of oxaloacetate and pyruvate. We interpret this difference as an indication that the oxidation of malate or of citrate saturates the capacity of the system to reoxidize reduced DPN, whereas the rate of respiration in the presence of oxaloacetate and pyruvate is determined by the rate of formation of citrate. Krebs & Kornberg (1957) have postulated that the rates of many metabolic sequences are determined by the low intermediate concentrations and not by the enzyme concentrations. This is applicable to the constitutive enzyme reactions of the citric acid cycle itself, but many of the supply lines in specialized tissues are likely to be controlled by enzyme concentrations.

Krebs & Bellamy (1960) have shown that the major pathway for glutamic acid utilization in a number of respiring tissues goes via transamination to oxaloacetate to form α-oxoglutarate and aspartate and concomitant oxidation of α-oxoglutarate to oxaloacetate.

The metabolism of glutamate by mammary-gland mitochondria appears to be straightforward. Respiration proceeds at about the same rate in the presence of glutamate or α-oxoglutarate, whereas the activity of glutamic dehydrogenase is negligible. It is therefore evident that transamination produces α-oxoglutarate from glutamate very rapidly. Since there is a block in the disposal of oxaloacetate, conditions should be favourable for transamination. This sequence of events should be most useful for the synthetic activity of the mammary gland as nitrogen is preserved for anabolic activities instead of being lost via an energy-requiring process.

In liver mitochondria, however, we have shown that the level of enzymic activity for glutamic dehydrogenase under optimum conditions is between 5 and 10 times that required to account for the oxygen consumption on addition of glutamate. The control between transamination and oxidation must rest with the level of α-oxolutarate concentration and the ratios of DPN/DPNH.

Using the data tabulated by Burton (1957) one can calculate the following equilibrium at pH 7 and 25°:

$$\frac{[\alpha\text{-Oxoglutarate}]\,[NH_4^+]\,[DPNH]}{[\text{Glutamate}]\,[DPN]} = 10^{-6\cdot28}\text{M}.$$

According to the data for the tissue concentration levels of the components of this reaction system (Krebs & Bellamy, 1960) no direct oxidation of glutamic acid is likely to occur *in vivo* under normal circumstances. In isolated and washed mitochondria a variety of situations can develop which depend on the endogenous concentrations and the accumulation of the various reactants during subsequent processes. On addition of glutamate to liver mitochondria the action of dehydrogenase and transaminase should rapidly produce enough α-oxoglutarate to inhibit the former enzyme.

The effect of malonate on the oxidation of glutamate by heart-muscle sarcosomes has been studied by Borst & Slater (1960), who find that no respiration occurs if the supply of oxaloacetate is cut off by the inhibition of succinate oxidation. In the presence of glutamate the oxygen uptake by guinea-pig-liver mitochondria was reduced to about a third on addition of malonate, whereas in mammary-gland mitochondria malonate inhibited respiration completely (Fig. 5). This indicates that some direct glutamate oxidation occurs in liver mitochondria. Mammary-gland mitochondria, with their block in malate oxidation due to accumulation of oxaloacetate, enable one to demonstrate the Borst & Slater effect in another way. Fig. 2 gives the record of oxygen uptake during an experiment in which malate is oxidized in the presence of malonate until the accumulation of oxaloacetate inhibits malate oxidation, at which point glutamate is added. Though it has been shown that glutamate is not oxidized in the presence of malonate (Fig. 5*B*), when oxaloacetate is present oxidation occurs. The lag in reaching optimum respiration rate on addition of glutamate is probably due to the inhibition of both succinate and malate oxidation by oxaloacetate and respiration is stimulated as oxaloacetate is converted into aspartate.

Liver mitochondria could oxidize glutamate directly, but it is not known under which physiological conditions they will do so. Mammary-gland mitochondria, on the other hand, are safeguarded from this wasteful disposal of a useful metabolite. The primitive control of metabolic pathways relying on the concentrations of various reactants for the choice of alternate metabolic routes may be of greater importance in the liver, where both anabolic and catabolic reactions are important and have to be balanced. The mammary gland on the other hand should have adapted its complement of enzymes for maximum efficiency of synthetic pathways.

SUMMARY

1. Methods for the preparation and criteria for the integrity of guinea-pig mammary-gland mitochondria are described, and the oxidation of a number of substrates is compared with that of liver mitochondria.

2. Oxidations of the citric acid cycle proceed at approximately the same rate in liver and mammary-gland mitochondria. However, whereas liver mitochondria respire on addition of oxaloacetate,

mammary-gland mitochondria do not; they are apparently unable to decarboxylate oxaloacetate at a sufficient rate. The glutamic-dehydrogenase activity of mammary-gland mitochondria is also negligible compared with the high activity of liver mitochondria.

3. The control of the metabolism of glutamic acid by liver and mammary-gland mitochondria presents a number of special and interesting features. The mammary gland appears to have control mechanisms which favour its synthetic activity by preserving metabolites and energy. These are discussed in the light of current ideas on the primitive control of metabolism.

We are indebted to the Rockefeller Foundation for a grant to the Physiology Department for the purchase of a Spinco preparative ultracentrifuge and to the Director, Microbiological Research Establishment, Porton, for the loan of a polarograph. It is a pleasure to acknowledge many helpful discussions with Dr S. J. Folley, F.R.S.

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Biosynthesis of C_4 Acids in *Pseudomonas fluorescens* KB1

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In recent years much has been learned about the mechanisms for the biosynthesis of C_4 acids in micro-organisms. The occurrence of fixation of carbon dioxide of the C_3+C_1 type has been demonstrated in baker's yeast (Davis, Cheldelin, Christensen & Wang, 1956; Stoppani, Conches, de Favelukes & Sacerdote, 1958), *Escherichia coli* (Abelson, Bolton & Aldous, 1952), and others. Fixation of carbon dioxide of the $C_1+C_2+C_1$ type was detected in *Rhodospirillum rubrum* (Cutinelli, Ehrensvard, Reio, Saluste & Sternholm, 1951), *Clostridium kluyveri* (Tomlinson, 1954) and *Penicillium digitatum* (Noble, Reed & Wang, 1958). Recently the operation of the malate-synthetase reaction (Wong & Ajl, 1956), a key step in the glyoxylate cycle, has been reported as playing an important role in acetate-grown *Pseudomonas fluorescens* KB1 and *E. coli* cells (Kornberg & Madsen, 1958). In the former organism, the fixation of carbon dioxide was also considered as an essential process in cellular biosynthesis (Kornberg & Quayle, 1958). More recently, the adaptive formation of both malate synthetase and *iso*citratase has been demonstrated by Reeves & Ajl (1960) in *E. coli* induced to utilize acetate as the sole carbon source.

In the present work the nature and the participation of major catabolic pathways functioning in proliferating cells of *P. fluorescens* KB1 were examined by an analysis of the rate and extent of $^{14}CO_2$ production from cells metabolizing specifically ^{14}C-labelled glucose samples. The fixation of carbon dioxide known to occur in this organism has been identified as the C_3+C_1 type. The C_3 component in the carbon dioxide-fixation process is believed to be the pyruvate derived from the degradation of glucose via the Entner–Doudoroff pathway, a predominant pathway for glucose catabolism in this organism. An estimation has been made of the relative contributions of fixation of carbon dioxide and of the malate-synthetase reaction towards the overall biosynthesis of C_4 acids in this organism.

EXPERIMENTAL

Culture and incubation procedures. The culture of *P. fluorescens* KB1 was kindly furnished by Dr H. L. Kornberg and has been kept on agar slants and in broth cultures.

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The culture medium employed in the present work was: glucose, 10 g.; $(NH_4)_2SO_4$, 3 g.; KH_2PO_4, 7 g.; NaCl, 1 g.; $MgSO_4$, 0·5 g.; Difco yeast extract, 0·5 g.; $MnSO_4$, trace; $FeSO_4$, trace; all were dissolved in 1 litre of water and the pH was adjusted to 6·7 with dilute NaOH solution. The inoculated culture, 100 ml. in a 500 ml. conical flask, was shaken at 30° for 16 hr., at which time the culture was in the middle of the exponential phase. The cells were harvested by centrifuging, washed thoroughly with medium free of carbon sources and resuspended in fresh medium without glucose. The concentration of cell suspension used for the subsequently described radiorespirometric and incorporation experiments was approximately 40 mg. in 100 ml. of medium. Incubation was at 30°.

Radiochemical substrates. The radiochemical substrates used in this work included [1-^{14}C]-, [2-^{14}C]-, [3-^{14}C]- and [6-^{14}C]-glucose, kindly furnished by Dr H. S. Isbell of the National Bureau of Standards. [3:4-$^{14}C_2$]Glucose was prepared in this laboratory according to the method of Wood, Lifson & Lorber (1945). Paper chromatography and radioautography were used to confirm the purity of the respective radiochemical substrates.

Radiorespirometric experiments. The radiorespirometric studies on the utilization of acetate and glucose by this organism were carried out according to the method of Wang *et al.* (1958). Usually 20 ml. of cell suspension, containing 0·4 mg. of cells/ml., was used for each incubation flask and the labelled substrate, in the amounts given in Table 1, was added from the side arm. At the end of the experiment, the cells and incubation media were separated by centrifuging and assayed for radioactivity.

Incorporation experiments. Incorporation experiments to trace the fate of glucose carbon atoms in cellular biosynthesis were carried out in the following manner: to 50 ml. of the cell suspension, containing 21·7 mg. of cells, was added a defined amount of specifically ^{14}C-labelled glucose (Table 2) and the culture was shaken at 28°. The progress of substrate utilization was followed by examining the recovery of radioactivity in respiratory CO_2 at regular intervals. When $^{14}CO_2$-recovery data indicated exhaustion of the substrate, 9 hr. in the present case (Fig. 1), the cells were harvested, washed with water and dried over P_2O_5 *in vacuo*.

The incorporation experiment designed to trace the role of CO_2 in cellular biosynthesis was carried out in a closed flask equipped with a rubber vial seal and two side arms designed for CO_2 generation and CO_2 absorption at the end of the experiment. To the flask containing 50 ml. of cell suspension, with 21·7 mg. of cells and 90 mg. of unlabelled glucose, was introduced 100 μc of $^{14}CO_2$ having a specific activity of 8·58 μc/μmole. The flask was shaken at 30° for 9 hr. and the experiment was terminated by the addition of 12 N-HCl to bring the pH of the medium to 1. Upon removal of CO_2 from the flask, the cells were harvested, washed and dried as already described.

The harvested cells (30 mg. dry wt.) in each of the incorporation experiments were hydrolysed separately with 2 ml. of 6 N-HCl in sealed Pyrex tubes for 20 hr. at 110°. The hydrolysates were dried *in vacuo* and the residue was dissolved in 3 ml. of water. A portion (300 μl.) of each of the hydrolysates was paper-chromatographed, with Whatman no. 1 filter paper and butan-2-ol–3% aq. NH_3 soln. (Roland & Gross, 1954), to separate individual amino acids. The radioactivity of individual amino acids on the paper chromatograms was determined by means of the liquid-scintillation counting technique described by Wang & Jones (1959).

To the major portion of each of the individual hydrolysates was added a prescribed amount (usually 2 g.) of unlabelled cell hydrolysate prepared in the same manner as the labelled hydrolysate. The amino acids in the mixed hydrolysates were separated by means of ion-exchange-resin column chromatography according to the method of Hirs, Moore & Stein (1954). The purity of each of the separated amino acids was established by means of paper chromatography and radioautography.

The samples of aspartic acid obtained by resin chromatography weighed 126·1, 129·7, 127·0 and 138·5 mg. from [1-^{14}C]glucose, [2-^{14}C]glucose, [6-^{14}C]glucose and $^{14}CO_2$ experiments respectively. The samples were assayed for specific activities and subsequently diluted 20-fold with unlabelled carrier before degradation studies. Aspartic acid samples (about 1 m-mole) were degraded in the following manner to obtain the specific activity of individual carbon atoms. (1) Ninhydrin decarboxylation to convert C-1 and C-4 into CO_2 (Van Slyke, MacFayden & Hamilton, 1943). (2) Aspartic acid was converted into malic acid with silver nitrite and HCl (Davis, 1954). The resulting malic acid, having its purity established by paper chromatography, was degraded by the Von Pechmann reaction (Recusen & Aronoff, 1953) to yield C-4 as CO_2 and C-1 as CO. The CO sample was converted into CO_2 by passing it through CuO at 400°. Malic acid was also oxidized to acetaldehyde by permanganate oxidation (Wood, Werkman, Hemingway & Nier, 1941). The acetaldehyde sample was further degraded by means of the iodoform reaction, forming CHI_3 from C-3 and formic acid from C-2. The formic acid was converted into CO_2 by HgO oxidation (Osburn, Wood & Werkman, 1933).

Determination of radioactivity. The respiratory CO_2 samples obtained in the radiorespirometric experiments were absorbed in 10 ml. of methanolic 0·25 M-hyamine hydroxide [(*p*-di*iso*butylcresoxyethoxyethyl)dimethylbenzyl ammonium hydroxide] and placed in a radiorespirometric CO_2 trap (Wang *et al.* 1958). A portion (5 ml.) of the methanolic solution was mixed with 10 ml. of toluene containing terphenyl (3 g./l.) and 1:4-bis-2(5-phenyloxazolyl)-benzene (30 mg./l.) in a 20 ml. glass counting vial. Countings were carried out by means of the Tricarb liquid-scintillation counter with photomultiplier voltages set at 1370 v and pulse discriminator set at 10–100 v and 10–∞ v in two channels. The cell and incubation medium samples collected in these experiments were also counted in the liquid-scintillation counter in the form of thixotropic-gel preparations according to the method of White & Helf (1956). The efficiency of counting with respect to each type of counting samples was determined by the use of internal standards.

The $^{14}CO_2$ samples obtained in the degradation experiments were converted into $BaCO_3$ and mounted on aluminium planchets by means of the centrifuging technique (Hutchens, Claycomb, Cathey & Van Bruggen, 1950). The radioactivity determinations were carried out with a gas-flow Geiger–Müller counter equipped with a thin Mylar window. Corrections for background and self-absorption were applied to the counting data in the conventional manner.

The counting data in the present work carried a standard deviation no greater than 2%.

RESULTS

The radiorespirometric data on the utilization of specifically ^{14}C-labelled glucose by *P. fluorescens* KB1 are given in Fig. 1. Table 1 shows the radioactivity distribution in respiratory CO_2, cells and incubation medium at the end of each of the experiments.

Table 2 gives the distribution of radioactivity among the carbon atoms of aspartic acid isolated

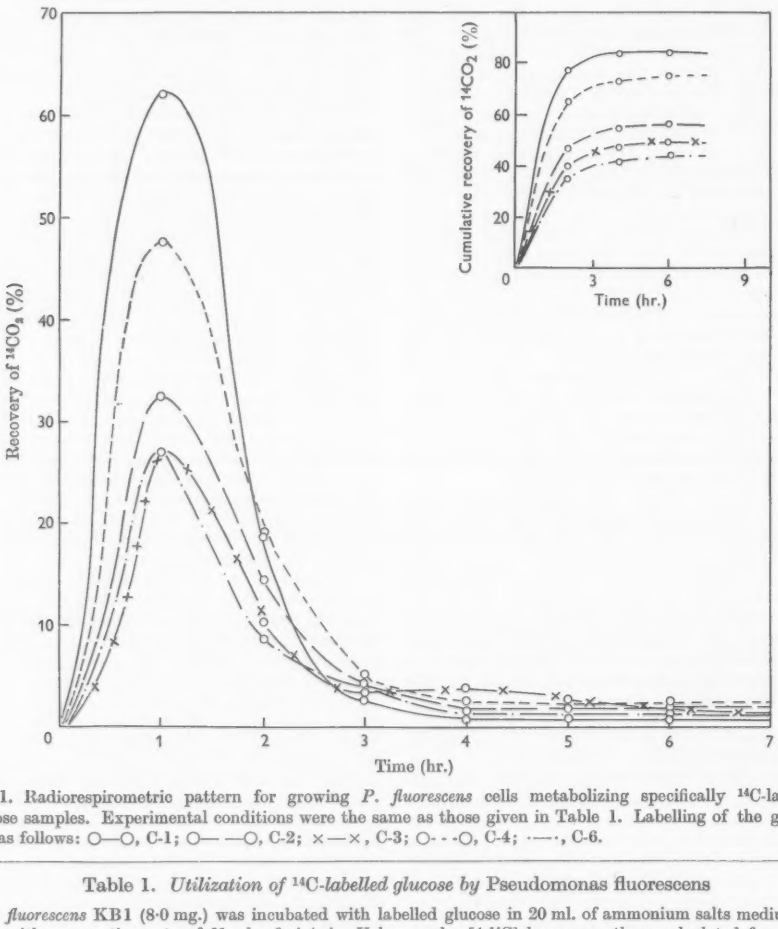

Fig. 1. Radiorespirometric pattern for growing *P. fluorescens* cells metabolizing specifically ^{14}C-labelled glucose samples. Experimental conditions were the same as those given in Table 1. Labelling of the glucose was as follows: O—O, C-1; O— —O, C-2; ×—×, C-3; O- - -O, C-4; ·—·, C-6.

Table 1. *Utilization of* ^{14}C*-labelled glucose by* Pseudomonas fluorescens

P. fluorescens KB1 (8·0 mg.) was incubated with labelled glucose in 20 ml. of ammonium salts medium for 7 hr. with an aeration rate of 61 ml. of air/min. Values under [4-^{14}C]glucose are those calculated from data obtained in [3-^{14}C]glucose and [3:4-$^{14}C_2$]glucose experiments.

	Substrate		Distribution of radioactivity (%)		
Substrate	Amount (mg.)	Radioactivity (μc)	Respiratory CO_2	Cells	Medium
[1-^{14}C]Glucose	10·0	0·27	84	8	2
[2-^{14}C]Glucose	10·0	0·24	56	32	6
[3:4-$^{14}C_2$]Glucose	10·0	0·12	65	28	5
[3-^{14}C]Glucose	10·0	0·06	52	42	6
[4-^{14}C]Glucose	—	—	78	14	4
[6-^{14}C]Glucose	10·0	0·44	47	38	7

Table 2. *Isotopic-distribution patterns of aspartic acid isolated from cells grown on $^{14}CO_2$ or specifically ^{14}C-labelled glucose samples*

P. fluorescens was grown in the presence of labelled CO_2 or glucose in 50 ml. of ammonium salts medium for 9 hr. Radioactivity incorporated into the various carbon atoms of aspartic acid was determined (see text).

Labelled substrate	[1-^{14}C]Glucose	[2-^{14}C]Glucose	[6-^{14}C]Glucose	$^{14}CO_2$
Cell wt., initial (mg.)	21·7	21·7	21·7	21·7
Cell wt., final (mg.)	33·0	47·3	43·5	54·5
Substrate: Glucose (mg.)	90	90	90	90
Substrate: Glucose (μc)	14·7	10·95	15·56	—
Substrate: CO_2 (μc)	—	—	—	100
$10^6 \times$ Respiratory CO_2 (counts/min.)	0·82	0·34	0·51	—

Aspartic acid	$10^6 \times$Specific activity (counts/min./m-mole)	Percentage of total	$10^6 \times$Specific activity (counts/min./m-mole)	Percentage of total	$10^6 \times$Specific activity (counts/min./m-mole)	Percentage of total	$10^6 \times$Specific activity (counts/min./m-mole)	Percentage of total
Whole molecule by combustion	1·77		1·99		2·01		16·2	
Whole molecule by summation	1·790	100	1·900	100	1·780	100	16·30	100
(1) CO_2H	1·080	60	0·537	28	0·394	22	1·46	9
(2) $CH(NH_2)$	0	0	0·635	34	0·432	24	0	0
(3) CH_2	0·021	1	0·245	13	0·563	32	0·16	1
(4) CO_2H	0·690	39	0·478	25	0·394	22	14·70	91

from cells grown on $^{14}CO_2$ or specifically ^{14}C-labelled glucose samples. The specific activities are corrected for variations in radioactivity of the original substrates.

DISCUSSION

The catabolic mechanisms for the utilization of glucose by the pseudomonads have been previously studied in many laboratories. It is generally recognized that these micro-organisms rely heavily on the operation of the Entner–Doudoroff pathway for the primary breakdown of glucose (Entner & Doudoroff, 1952; Stern, Wang & Gilmour, 1960). In several species of pseudomonads (Stern *et al.* 1960; Kogut & Podoski, 1953) the degradation product of glucose, such as pyruvate, is known to be degraded further by way of the tricarboxylic acid cycle, giving rise to various intermediates for biosynthetic functions and eventually to carbon dioxide for energy production. *P. fluorescens* KB1 is known to be equipped with tricarboxylic acid-cycle enzymes (Kogut & Podoski, 1953) and it can grow on either glucose or acetate as the sole carbon source. With cells grown on acetate, the operation of the malate-synthetase reaction has been recently reported by Kornberg & Madsen (1958), and carbon dioxide may have played a role in the biosynthesis of C_4 acids in this organism (Kornberg & Quayle, 1958).

With specifically ^{14}C-labelled glucose samples as substrates, it is found that the radiorespirometric data given in Fig. 1 and Table 1 resemble closely those reported for other species of pseudomonads, particularly *P. reptilivora* and *P. aeruginosa* (Stern *et al.* 1960). Qualitatively, the important role played by the Entner–Doudoroff pathway in the overall catabolism is clearly indicated by a comparison of $^{14}CO_2$ recovery from various carbon atoms of glucose, i.e.

$$C\text{-}1 > C\text{-}4 > C\text{-}2 > C\text{-}3 \geqslant C\text{-}6.$$

The order is that to be expected from the operation of the Entner–Doudoroff pathway, by which essentially 2 moles of pyruvate are formed from glucose with their methyl carbon atoms equivalent to C-3 and C-6, carbonyl carbon atoms equivalent to C-2 and C-5 and carboxyl carbon atoms equivalent to C-1 and C-4 of glucose. The fact that $^{14}CO_2$ recovery from C-1 of glucose is significantly greater than that from C-4 revealed the occurrence of a pathway other than the classical Entner–Doudoroff pathway. The nature of this alternate pathway is presumably that involving the decarboxylation of phosphogluconate, giving rise to a mole each of carbon dioxide and of pentose phosphate (Lewis, Blumenthal, Weinrach & Weinhouse, 1955; Wood, 1955). The $^{14}CO_2$ recovery from C-3 is practically identical in rate and magnitude with that from C-6. This fact may mean that the pentose phosphate, formed in the hexose monophosphate pathway, is not engaged extensively in further respiratory activities.

Estimation of pathway participation, according to the method of Wang *et al.* (1958) and by the use of the radiorespirometric data observed when the organism had utilized all the administered substrate in the incubation medium, revealed that approximately 9% of administered glucose was

catabolized by way of the phosphogluconate decarboxylation pathway. The remaining 91% was catabolized by way of the Entner–Doudoroff pathway, with the formation of pyruvate.

To trace the fate of pyruvate participating in the biosynthetic functions, isotopic-distribution patterns of aspartate samples isolated from cells grown on $^{14}CO_2$ and specifically ^{14}C-labelled glucose samples were obtained by degradation studies. The net synthesis of cellular constituents by cells in the incorporation experiments is evidenced by the observed gain in cell weights shown in Table 2. This fact implies that the labelling data of aspartic acid given in Table 2 represents essentially that derived from net incorporation rather than that from exchange reactions. Moreover, in view of the recognized precursor–product relationship between aspartate and other C_4 acids such as oxaloacetate or malate, the findings with aspartate should help in understanding the biosynthesis of C_4 acids in

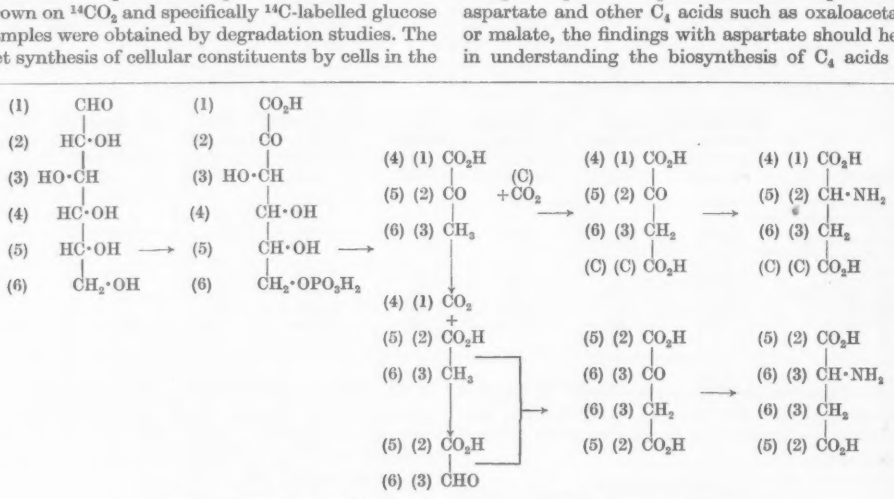

Fig. 2. Biosynthetic pathways for aspartic acid in *P. fluorescens* KB1.

Table 3. *Isotopic-distribution patterns of aspartate from various pathways*

Pathway followed by the labelled carbon atoms	Expected and observed isotopic-distribution pattern (%)			
	HO_2C	CH_2	$CH(NH_2)$	CO_2H
$^{14}CO_2$				
Via CO_2 fixation (C_1+C_3)	100	0	0	0
Observed	91	1	0	9
[1-^{14}C]Glucose				
Via Entner–Doudoroff route and CO_2 fixation	0	0	0	100
Via Entner–Doudoroff and malate-synthetase reactions	0	0	0	0
Observed	39	1	0	60
[2-^{14}C]Glucose				
Via Entner–Doudoroff route and CO_2 fixation	0	0	100	0
Via Entner–Doudoroff and malate synthetase reactions	50	0	0	50
Via Entner–Doudoroff route, CO_2 fixation and tricarboxylic acid cycle				
One turn	25	25	25	25
Extensive	50	0	0	50
Observed	25	13	34	28
[6-^{14}C]Glucose				
Via Entner–Doudoroff route and CO_2 fixation	0	100	0	0
Via Entner–Doudoroff route and malate-synthetase reactions	0	50	50	0
Via Entner–Doudoroff route, CO_2 fixation and tricarboxylic acid cycle				
One turn	25	25	25	25
Extensive	16·7	33	33	16·7
Observed	22	32	24	22

general. It should be noted though that the experiment with carbon dioxide, although informative, is not to be compared directly with the glucose experiments.

With glucose as substrate one finds that the specific activities of aspartate samples are comparable in magnitude with each of the labelled glucose substrates. This fact led us to believe that an intact C_3 unit, presumably in the nature of pyruvate, is involved in the major biosynthetic pathway for aspartic acid. That the latter pathway is related directly to a fixation of carbon dioxide of the C_1+C_3 type is suggested by the heavy labelling of C-4 of aspartate from environmental $^{14}CO_2$.

As a help in following the contribution of each pathway to the synthesis of C_4 acids, the major pathways suspected to occur in this organism, namely fixation of carbon dioxide of the C_1+C_3 type and malate-synthetase reaction, are shown in Fig. 2. The expected isotopic-distribution patterns of aspartate from the operation of each of these pathways as well as the observed patterns are given in Table 3. Perfect agreement between the expected pattern and the observed pattern would not be expected in view of the possible operation of several mechanisms for labelling randomization. Nevertheless, important evidence for the operation of fixation of carbon dioxide of the carbon dioxide+pyruvate type is provided by the findings in the $^{14}CO_2$ and [1-^{14}C]glucose experiments. The detection of small amounts of $^{14}CO_2$ activity (9%) in C-1 of aspartate is presumably the result of randomization of labelling via equilibration with fumarate. If one could use the latter figure as an index for the extent of labelling randomization, it is then possible to conclude that the heavy labelling of C-1 of aspartate from C-1 of glucose represents primarily a direct incorporation of pyruvate carboxyl carbon atoms. The observed minor labelling in C-4 of aspartate from C-1 of glucose probably reflects partially the randomization of labelling and partially the re-entry of metabolic carbon dioxide via the carbon dioxide-fixation process. The latter is possible in view of the magnitude of the specific activity of the respiratory carbon dioxide in the [1-^{14}C]glucose experiment.

The findings in the experiments with [2-^{14}C]-glucose and [6-^{14}C]glucose agree at least qualitatively with those expected from the sequential reactions involving the fixation of carbon dioxide and the tricarboxylic acid-cycle processes described above. The exact nature of the fixation of carbon dioxide reaction cannot be deduced from the present findings, but it could be either the carboxylation of phosphoenolpyruvate (Utter & Kurahashi, 1954) or the reaction catalysed by the malic enzyme (Ochoa, Mehler & Kornberg, 1948).

With the present findings, although it is impossible to rule out the contribution of malate-synthetase reaction in C_4 synthesis, it is reasonable to speculate that the malate-synthetase reaction, if it occurs, does not play a major role in the biosynthesis of C_4 acids in glucose-grown cells.

SUMMARY

1. Evidence is presented that the biosynthesis of aspartic acid in glucose-grown *Pseudomonas fluorescens* KB1 cells relies heavily, if not exclusively, on the operation of fixation of carbon dioxide of the C_1+C_3 type.

2. The C_3 unit, presumably of the nature of pyruvate, is believed to be derived from glucose via the Entner–Doudoroff pathway, which accounts for the catabolism of nine-tenths of the substrate glucose in this organism.

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The Metabolism of Naphthalene and 1-Naphthol by Houseflies and Rats

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This report describes a study in which houseflies, *Musca domestica*, were fed with radioactive naphthalene or radioactive 1-naphthol and the resulting excretory products compared with those produced by rats. Kikal & Smith (1958) reported the excretion of free phenols and their cysteine conjugates by locusts given chlorobenzene. Friedler & Smith (1954) reported that locusts conjugate aromatic acids with glycine and Smith (1955) found that ethereal sulphates and glucosides were metabolic products. The present paper shows that houseflies metabolize naphthalene in a manner similar to rats but that 1-naphthol follows slightly different pathways of metabolism in the two species.

MATERIALS AND METHODS

Experiments with houseflies. Flies (3–4-day old) of mixed sexes were used. The compounds were administered by incorporation into the feed, a 5% sucrose solution. One-gallon glass jars, equipped with tight lids and a dehydrating agent, were used as containers for the flies. The jars were kept sealed for 8–10 hr. after adding the test compounds in order to reduce losses due to volatility of the radioactive material. The radioactive naphthalene and 1-naphthol were fed at the rate of approximately 25 μg./day until from 50 to 150 μg. had been used. Approximately 150 flies were used in each test. The specific activities of the radioactive compounds used were 2·0 mc/m-mole for naphthalene and 2·3 mc/m-mole for 1-naphthol. Neither of the compounds was toxic at the amounts fed and the mortality in the control and treated insects was parallel, about 50% after 4 days. No attempt was made to eliminate dead flies during the isolation of radioactivity. At the end of a feeding period, 4–8 days, both dead and living flies were ground with anhydrous sodium sulphate in a mortar and pestle and extracted in a Soxhlet apparatus with ethanol. The excreted material in the feeding jars was extracted with 80% ethanol.

Experiments with rats. Each of three 50 g. weanling rats, one male and two females, was given approximately 70 μg. of radioactive naphthalene or 1-naphthol intraperitoneally in peanut oil. Urine was collected after 48 hr., extracted with ether and then with butan-1-ol and the two extracts were chromatographed.

Paper chromatography. The solvent systems used were: (1) butan-1-ol–ethanol–water (17:3:20, by vol.) (Boyland & Solomon, 1956); (2) butan-1-ol–3N-$(NH_4)_2CO_3$–aq. 3N-NH_3 soln. (4:3:3, by vol.) (Corner & Young, 1955). Both ascending and descending techniques on Whatman no. 1 paper were used with no significant differences in R_F values. The developed chromatograms were examined for their radioactive spots with a gas-flow windowless scanning device and the measured radioactivity was recorded on charts synchronized with the scanning head. Occasional chromatograms were confirmed by radioautography on X-ray film.

Reference compounds. 1:2-Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid, *N*-acetyl-*S*-(1:2-dihydro-2-hydroxynaphthyl)cysteine (1-naphthylpremercapturic acid), 2-hydroxy-1-naphthyl sulphate and *N*-acetyl-*S*-(1-naphthyl)cysteine (1-naphthylmercapturic acid) were kindly supplied by Professor E. Boyland. 1:2-Dihydro-1:2-dihydroxynaphthalene was isolated from rat urine by the method of Corner & Young (1955) and 1-naphthyl glucosiduronic acid by the method of Berenbom & Young (1951). 1-Naphthyl sulphate was synthesized by the method of Feigenbaum & Neuberg (1941).

RESULTS

When the excreta of [1-^{14}C]naphthalene-fed houseflies were examined by paper chromatography with solvent system (1), seven to nine and occasion-

ally ten radioactive substances appeared to be present. With solvent (2), up to nine radioactive spots could be detected. Similar results were obtained when the flies were fed with 1-[1-^{14}C]-naphthol. The urine of naphthalene-treated rats contained the same metabolites except for 1-naphthyl glucosiduronic acid, which was absent, with the conjugates appearing in the butanol extract and 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene appearing in the ether extract. The urine of 1-[1-^{14}C]naphthol-treated rats contained six metabolites. Table 1 gives the R_F values found.

When houseflies were fed with sodium [^{35}S]-sulphate and non-radioactive naphthalene, radioactive excretory products with R_F 0·55 and 0·69 (solvent 1) and R_F 0·53 and 0·81 (solvent 2) were obtained. These were not produced by flies receiving only radioactive sodium sulphate. The compounds were probably *N*-acetyl-*S*-(1-naphthyl)cysteine and 1-naphthyl sulphate respectively.

In typical experiments with naphthalene-treated houseflies, zones corresponding to R_F (solvent 1) 0·07, 0·13 and 0·24 were excised from chromatograms and eluted with water. One portion of each eluate was incubated with β-glucuronidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) for 24 hr. at 37° in 0·075 M-phosphate buffer, pH 6·8. A portion without enzyme served as control. The activity of the enzyme was confirmed by the hydrolysis of phenolphthalein glucosiduronide. The incubated mixtures were extracted with ether and chromatograms of the extract were developed in solvent (1). The chromatogram of the product R_F 0·07, thought to be 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid, exhibited two radioactive spots with the same R_F values as 1:2-dihydro-1:2-dihydroxynaphthalene and 1-naphthol. The zone R_F 0·13, assumed to be 1-naphthyl glucosiduronic acid, yielded 1-naphthol. The zone R_F 0·24, thought to be 1:2-dihydro-1-naphthyl glucosiduronic acid, yielded radioactivity at the origin after spotting but none after development. An explanation might be that hydrolysis of this glucosiduronide would result in the production of naphthalene, which would disappear from the paper upon drying (Boyland & Solomon, 1955).

The metabolite R_F 0·38, thought to be 1-naphthylpremercapturic acid, was eluted from its chromatogram, spotted again on fresh paper, exposed to HCl fumes for 1 hr. at room temperature and developed in solvent (1). Under these conditions radioactive products occurred at R_F 0·94 (1-naphthol). When the R_F 0·38 spot was treated with 1 N-HCl for ½ hr., the developed chromatogram exhibited radioactivity at R_F 0·55 (1-naphthylmercapturic acid) and R_F 0·94 (1-naphthol). In a similar test with HCl fumes, the material found in the region R_F 0·52–0·56 was hydrolysed to 1-naphthol.

Table 1. *R_F values of metabolites produced by houseflies and rats after treatment with* [1-^{14}C]*naphthalene and* 1-[1-^{14}C]*naphthol*

Solvent systems: (1) butan-1-ol–ethanol–water (17:3:20) (15 hr.); (2) butan-1-ol–3 N-$(NH_4)_2CO_3$–aq. 3 N-NH_3 soln. (4:3:3) (15 hr.). Solvents are indicated by numbers at heads of columns.

	R_F				Urine of rats given		Excreta of houseflies given			
	Standards		Literature		[1-^{14}C]-Naphthalene	1-[1-^{14}C]-Naphthol	[1-^{14}C]-Naphthalene		1-[1-^{14}C]-Naphthol	
Metabolites	(1)	(2)	(1)	(2)	(1)	(1)	(1)	(2)	(1)	(2)
1:2:Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid	0·07	0·09	0·08*	0·09†	0·06	0·07	0·07	0·09	0·06	0·05
1-Naphthyl glucosiduronic acid	0·12	0·22	0·15*	0·22†	—	0·17	0·13	0·22	0·17	0·26
1:2-Dihydro-1-naphthyl glucosiduronic acid	—	—	0·22*	—	0·26	—	0·24	—	0·24	—
N-Acetyl-*S*-(1:2-dihydro-2-hydroxynaphthyl)cysteine	0·39	0·43	0·38‡	—	0·36	—	0·38	—	0·35	—
2-Hydroxy-1-naphthyl sulphate	0·56	0·82	0·45§	—	0·43	0·44	0·45	—	0·43	—
N-Acetyl-*S*-(1-naphthyl)cysteine	0·56	0·51	0·55*	—	0·52	0·53	0·54	0·53	0·54	0·51
1-Naphthyl sulphate	0·68	0·78	0·69*	0 81†	0·65	0·66	0·66	0·80	0·65	0·80
1:2-Dihydro-1:2-dihydroxy-naphthalene	0·86	0·88	0·86*	0·90†	0·85	—	0·86	0·91	0·84	0·90
1-Naphthol	0·93	0·95	0·96*	—	0·94	0·94	0·93	0·95	0·93	0·95
Unidentified	—	—	—	—	0·75	—	0·77	—	0·73	—
Unidentified	—	—	—	—	—	—	—	0·13, 0·32 0·65	—	0·13, 0·37 0·66

* Boyland & Solomon (1956).
† Corner & Young (1955).
‡ Boyland & Sims (1958).
§ Boyland & Sims (1957).

The metabolite R_F 0·45 hydrolysed by exposure to HCl fumes as described above produced spots of R_F 0·01, 0·82 and 0·94, the last two regions corresponding to 1:2-naphthaquinone and 1-naphthol respectively. The spot at the origin is unidentified but has been observed whenever 1:2-dihydroxynaphthalene or 1:2-naphthaquinone is chromatographed in solvent (1). The results suggest that the metabolite was 2-hydroxy-1-naphthyl sulphate. The reference compound provided by Professor E. Boyland, however, had R_F 0·56.

In solvents (1) and (2), 1- and 2-naphthol have the same R_F but in aq. 0·1N-NH_3 soln. they are separated (Boyland & Sims, 1958). With this solvent it was shown that the radioactive material in the R_F 0·93–0·96 region of systems (1) and (2) was 1-naphthol and that 2-naphthol was not present in excreta of either houseflies or rats treated with naphthalene.

The metabolite of R_F 0·84 from 1-naphthol-treated houseflies was eluted, chromatographed again, eluted a second time, spotted, exposed to HCl fumes and developed in aq. 0·1N-NH_3 soln. The product had the R_F of 1-naphthol, suggesting that the original radioactivity was probably present as 1:2-dihydro-1:2-dihydroxynaphthalene (Young, 1947). In agreement with Berenbom & Young (1951) and Boyland & Wiltshire (1953), the diol could not be detected in the urine of rats treated with 1-naphthol.

The unidentified material found in the region R_F 0·73–0·77 (solvent 1) yielded on acid hydrolysis radioactive spots at R_F 0·04, 0·77 and 0·93 (1-naphthol). This material occurred in both rat and fly experiments with naphthalene and in fly experiments with 1-naphthol.

Houseflies were given a single injected dose of [1-^{14}C]naphthalene or 1-[1-^{14}C]naphthol followed by a determination of radioactivity in the tissues and excreta of survivors by the Van Slyke wet-combustion method (Van Slyke, Steele & Plazin, 1951). After 24 hr. 7 % of the radioactivity from the naphthalene injection remained in the tissues of the survivors and 34 % was found in the excreta. With 1-naphthol the values were 10 and 43 % respectively. Expired CO_2 was not collected in these tests.

DISCUSSION

On the basis of results from 15 species of insects, Smith (1955) concluded that the conversion of phenolic substances into glucosides rather than glucosiduronides was a characteristic of insects. This conclusion is not supported by the present study of species of flies which were not included in Smith's survey.

There seems little doubt that houseflies produce all of the metabolites of naphthalene which are known to be excreted by rats. It also appears that 1-naphthol and naphthalene are converted by this insect into the same excretory products. There does seem to be a difference between houseflies and rats in the metabolism of 1-naphthol. Only six metabolites were excreted by the naphthol-treated rats, whereas nine and possibly ten were produced by flies given this compound.

Berenbom & Young (1951) and Boyland & Wiltshire (1953) have concluded that rats and rabbits receiving 1-naphthol produce 1-naphthol, 1-naphthyl sulphate and 1-naphthyl glucosiduronic acid. Two of the additional metabolites which we have found in rats, 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid and 1-naphthylmercapturic acid, may be the result of conjugations involving 1:2-dihydro-1:2-dihydroxynaphthalene, which has not been identified as a product of 1-naphthol metabolism in rats. The failure to detect 1-naphthylpremercapturic acid in these experiments may be due to its instability since no precautions were taken to prevent its conversion into 1-naphthylmercapturic acid.

SUMMARY

1. Houseflies and rats have been treated with [1-^{14}C]naphthalene and 1-[1-^{14}C]naphthol and the products of their metabolism examined.

2. By paper chromatography, these products have been identified as: glucosiduronides of 1-naphthol, 1:2-dihydro-1:2-dihydroxynaphthalene and 1:2-dihydro-1-hydroxynaphthalene; 1-naphthylsulphate; mercapturic acid conjugates of 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene; free 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene. 2-Hydroxy-1-naphthyl sulphate may also be present.

3. All of the metabolites produced by naphthalene- and 1-naphthol-treated flies are found in the urine of rats treated with naphthalene, but only six are produced by rats given 1-naphthol.

4. Metabolites not previously detected in the urine of 1-naphthol-treated rats are 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid and 1-naphthylmercapturic acid.

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Further Purification and Properties of the Amine Oxidase of Pea Seedlings

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Although amine oxidases have been studied intensively for more than 20 years, the nature of their prosthetic groups has not been established. The amine oxidase of pea seedlings belongs to the group of amine oxidases that is inhibited by carbonyl reagents such as hydrazine and semicarbazide; the group also includes the spermine, benzylamine and diamine oxidases of animal tissues. The sensitivity of animal diamine oxidase to carbonyl reagents led to the suggestion that the enzyme contains a functional aldehyde or ketone group (Zeller, 1938). Much evidence has since been reported that the prosthetic groups both of animal diamine oxidase (Davison, 1956; Goryachenkova, 1956) and of the related plant enzyme (Werle & Pechmann, 1949; Goryachenkova, 1956) contain pyridoxal phosphate. It has also been suggested that the prosthetic group of spermine oxidase contains pyridoxal phosphate (Tabor, Tabor & Rosenthal, 1954).

Hydrogen peroxide is a product of the reactions catalysed by amine oxidases, so it has been suggested that some of these enzymes are flavoproteins. Kapeller-Adler (1949) and Goryachenkova (1956) confirmed and extended the work of Zeller, Stern & Wenk (1940) and Swedin (1943, 1944) on the flavoprotein nature of animal diamine oxidase and concluded that the prosthetic group of the enzyme contains flavinadenine dinucleotide. Goryachenkova (1956) also reported evidence that flavinadenine dinucleotide forms a part of the prosthetic group of the amine oxidase of pea and clover seedlings.

The finding that animal diamine oxidase is inhibited by cyanide suggested that the enzyme is a metalloprotein. Zeller (1940) found that other reagents that react with heavy metals, such as sodium azide, thiourea and sodium sulphide, caused only small inhibitions, which were attributed to effects on secondary oxidations. It was concluded that animal diamine oxidase was inhibited by cyanide because this compound acts as a carbonyl reagent. The inhibition of the related plant enzyme by cyanide was attributed to the same cause by Kenten & Mann (1952). Mann (1955), working with 300-fold purified preparations of pea-seedling amine oxidase, found that the enzyme was inhibited by sodium diethyldithiocarbamate, salicylaldoxime and potassium ethyl xanthate and suggested that it might be a metalloprotein. Preliminary investigations of the heavy-metal content of the preparations showed that copper and manganese were present in possibly significant amounts. Werle & Hartung (1956) found that both the plant enzyme and animal diamine oxidase are inhibited by sodium diethyldithiocarbamate, salicylaldoxime, 8-hydroxyquinoline and dithizone. In the experiments on animal diamine oxidase, with 1:5-diaminopentane as substrate, the ammonia formation was completely inhibited by 8-hydroxyquinoline and sodium diethyldithiocarbamate. Such inhibitions cannot be attributed to effects on secondary oxidations. Suzuki (1959) reported that the plant enzyme is also inhibited by 2:2′-dipyridyl and 1:10-phenanthroline and, on the basis of the inhibition by metal-chelating agents, suggested the presence of iron, molybdenum or zinc in the prosthetic group of the enzyme.

The evidence so far obtained suggests the possibility that the enzymes contain pyridoxal phosphate, flavinadenine dinucleotide and a heavy metal in the prosthetic groups. Direct evidence for

pyridoxal phosphate or flavinadenine dinucleotide should be given by the absorption spectra of sufficiently purified preparations. Though the diamine oxidase and spermine oxidase of animal tissues have been extensively purified, no such spectrophotometric evidence has been reported. Pea seedlings provide a very rich source of plant amine oxidase. Mann (1955) described a method giving a 300-fold purification of the amine oxidase of pea-seedling extracts. The preparations so obtained were about 25 times as active, in the oxidation of 1:4-diaminobutane, as the purest preparations of animal diamine oxidase previously obtained. This suggested that evidence of the nature of the prosthetic group might be obtained more readily with the plant enzyme than with the related animal enzymes. The present work was started to test this possibility. A chromatographic procedure for further purifying the plant enzyme is reported together with preliminary spectrophotometric studies of the preparations and estimations of their content of heavy metals. Some of this work has already been briefly described (Mann, 1960).

MATERIALS AND METHODS

Calcium phosphate. Calcium phosphate (hydroxyapatite) was prepared by the method of Tiselius, Hjertén & Levin (1956).

Buffers. Sodium phosphate buffers were prepared from Na_2HPO_4 and H_3PO_4. Potassium phosphate buffers were prepared from KH_2PO_4 and KOH.

Catalase. Crystalline ox-liver catalase was prepared as described by Clarke & Mann (1959).

Estimation of amine oxidase. Amine oxidase was estimated manometrically, in air, in the Warburg apparatus at 28°. The volume of the reaction mixtures was 3 ml. and 0·2 ml. of 5N-KOH was present in the centre well. A unit of amine oxidase is defined as the amount which at 28° gives an uptake of 1 μl. of O_2/hr. (calculated from initial rates) in the presence of 10 mM-1:4-diaminobutane, catalase and 67 mM-phosphate buffer, pH 7, in a total volume of 3 ml. (Mann, 1955). The specific activity is defined as oxidase units/mg. of N.

Estimation of total nitrogen. The total nitrogen of the amine-oxidase preparations was estimated by a micro-Kjeldahl method. Samples (0·1–0·2 ml.) were digested with 1 ml. of conc. H_2SO_4 and approx. 0·2 g. of a catalyst (composition: K_2SO_4, 80 g.; $CuSO_4$,$5H_2O$, 10 g.; SeO_2, 1 g.). The ammonia was distilled into boric acid solution (1%) and estimated by titration with HCl.

Metal estimations. The manganese and copper contents of the amine-oxidase preparations were estimated colorimetrically. For each estimation a sample (2–4 ml.) was digested with 2 ml. of a mixture (1:1, v/v) of conc. H_2SO_4 and redistilled HNO_3. The manganese content of the digests was estimated by oxidation, with KIO_4, to permanganate (Sandell, 1950); copper was estimated with sodium diethyldithiocarbamate by the procedure of Forster (1953), except that the extinction of the final solutions was measured at 436 mμ. The metal contents of two preparations were estimated spectrographically by Dr H. H. Le Riche, Soil Survey, Rothamsted Experimental Station, Harpenden, Herts. Samples (4–5 ml.) of the enzyme preparations were dialysed against 2 l. of distilled water for about 15 hr. The dialysed enzyme was washed from the sac into a platinum crucible and mixed with 5 ml. of aq. $AlCl_3$ soln. (equivalent to 20 mg. of Al_2O_3). The $AlCl_3$ used was free from spectrum lines of the trace elements under investigation. A few drops of aq. NH_3 soln. (sp.gr. 0·88) were added and the mixture was evaporated to dryness at 100° and then ignited in a muffle furnace at 450°. The residue was analysed with a Hilger large spectrograph with a d.c. carbon arc.

Spectrophotometry. Absorption was measured with a Unicam SP. 500 spectrophotometer. Anaerobic conditions, where necessary, were established by using Thunberg-type assemblies fitted to silica cells by ground-glass joints. These were specially made by Unicam Instruments Ltd.

Preparation and purification of the enzyme

Initial preparations. The amine-oxidase preparations used as starting material for purification by chromatography were made from 9-day-old pea seedlings by the method of Mann (1955), except that the roots of the seedlings were discarded. The weight of plant material used for each preparation was 1–3 kg. The volume of the enzyme preparations (in 10 mM-potassium phosphate buffer, pH 7) was such that 1 ml. was equivalent to 100 g. of the plant tissue. These enzyme preparations were stored at −10° until there was enough for the next stage in the purification.

Chromatography on calcium phosphate (hydroxyapatite). All manipulations during the chromatography of the enzyme on hydroxyapatite and in the subsequent stages of the purification were carried out at 0–5°. Five to eight of the initial oxidase preparations were combined and centrifuged. A small sample of the supernatant solution was reserved for initial estimations and the rest was applied to a hydroxyapatite column (4 cm. × 12 cm.) equilibrated with 10 mM-potassium phosphate buffer, pH 7. Elution was started with 40 mM-potassium phosphate buffer, pH 7, and continued by stepwise increments (40 mM) in the concentration of buffer up to a final concentration of 200 or 240 mM. The volume of buffer at each concentration was 100 ml. The rate of flow varied from 50 to 80 ml./hr. in different experiments. The eluate was collected in 5 ml. fractions. The fractions of the main amine-oxidase peak showing the highest amine-oxidase activity in relation to $E_{280\ m\mu}$ were combined and dialysed against 2 × 3 l. of distilled water. The ratio oxidase units/$E_{280\ m\mu}$ in the fractions combined generally exceeded 10^4; fractions in which this ratio was less than 5×10^3 were rarely included.

Adsorption of inert material on aminoethyl- or diethylaminoethyl-cellulose. The dialysed oxidase solution was passed through a column (2 cm. × 10 cm.) of aminoethyl-cellulose (AE-cellulose) or diethylaminoethylcellulose (DEAE-cellulose), which was then washed with 25 ml. of 10 mM-potassium phosphate buffer, pH 7. To prepare the columns, Whatman AE 50 or DE 50 powder was suspended in water and adjusted to pH 7 with M-KH_2PO_4. The columns were equilibrated with 10 mM-potassium phosphate buffer, pH 7, before use.

Concentration on calcium phosphate (hydroxyapatite). The enzyme solutions and washings from the AE- or DEAE-cellulose column were combined and applied to a column of

Table 1. *Further purification of the amine oxidase of pea seedlings*

	Initial enzyme preparation				Final enzyme preparation				
	Amine oxidase				Amine oxidase				
Preparation no.	Vol. (ml.)	10^{-3} × Content (units/ml.)	N content (mg./ml.)	10^{-3} × Specific activity	Vol. (ml.)	10^{-3} × Content (units/ml.)	N content (mg./ml.)	10^{-3} × Specific activity	Yield (%)
1	150	40	0·450	89	16	185	0·970	191	59
2	80	61	0·625	98	10	258	1·100	235	53
3	120	46	0·438	105	20	153	0·700	219	55
4	130	54	0·500	108	14	254	0·775	328	51
5	155	43	0·575	75	20	187	0·675	277	56
6	160	50	0·514	97	14	250	0·900	278	44

hydroxyapatite (4 cm. × 4 cm.). The enzyme was eluted with 200 mM-potassium phosphate buffer, pH 7, and the active fractions of the eluate were combined and stored at −10°.

RESULTS

Purification of the enzyme

Chromatography on calcium phosphate (hydroxyapatite). Fig. 1 shows the fractionation of an initial enzyme preparation on the hydroxyapatite column (preparation 3, Table 1). Determinations of $E_{280\ m\mu}$ of the fractions suggested that the proteins were partially separated into three main parts. The first two (fractions 26–70 and 70–106) were yellow and the third (fractions 106–140) was weakly pink. The secondary peak among the pink fractions was generally, though not always, observed and was probably correlated with an increase in the concentration of buffer. The fractions round the main peak (fractions 114–118) were the pinkest. In the fractions on either side of this peak the colour decreased and was progressively masked or replaced by yellow. The bulk of the enzyme was found in the pink fractions. The curve relating enzyme units to fraction number showed major and minor peaks which coincided with those of the extinction curve. The fractions of the main peak (in this experiment fractions 96–124) were combined for later purification and concentration.

Adsorption of inert material on aminoethyl- or diethylaminoethyl-cellulose. Under the experimental conditions the enzyme in the dialysed combined active fractions from the hydroxyapatite column was not adsorbed on AE- or DEAE-cellulose but much inert material, including most of the yellow pigment, was adsorbed and separated from the enzyme, particularly in the later experiments (preparations 4–6, Table 1) in which DEAE-cellulose was used.

Concentration on calcium phosphate (hydroxyapatite). When the combined active fractions (300–400 ml.) from the AE- or DEAE-cellulose columns were applied to a hydroxyapatite column (4 cm. × 4 cm.) the enzyme was associated with a pink band, a few millimetres in depth, which

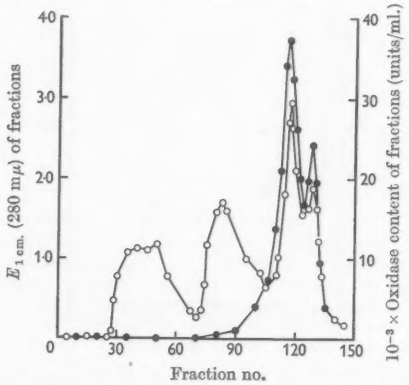

Fig. 1. Fractionation of an initial amine oxidase (preparation 3, Table 1) on a hydroxyapatite column. ○, $E_{1\ cm.}$ (280 mμ) of fractions; ●, amine-oxidase content of fractions.

formed at the top of the column. On elution with 200 mM-potassium phosphate buffer, pH 7, this band moved down the column and when it approached the bottom of the column the eluate was collected in 2 ml. fractions. The enzyme was found in the pink fractions, which were combined and stored at −10°.

Overall purification. Table 1 shows the amine-oxidase and total-N contents of the initial and final preparations from which the specific activities and yields were calculated. The specific activities of the amine oxidase in eight of the pea-seedling extracts from which the initial preparations of Table 1 were made ranged between 240 and 430, with an average value of 335. The average specific activity of the initial preparations of Table 1 was 94×10^3. This was increased to an average specific activity of 215×10^3 in preparations 1–3, where fractionation on hydroxyapatite was followed by fractionation on AE-cellulose, and to 294×10^3 in preparations 4–6, where DEAE- replaced AE-cellulose. The total purification of the amine oxidase

of the pea-seedling extracts in the latter preparations was therefore about 880-fold. The preparations were stable; activity was not clearly decreased after storage for several weeks at −10°.

Spectrophotometric investigations

Absorption spectrum. Fig. 2 shows the absorption spectrum of the purest of the final preparations (4, Table 1). The pink colour of the preparation was associated with a band with maximum absorption at about 500 mμ. This band was shown clearly by the undiluted preparation but it was very weak compared with the protein band at about 280 mμ ($E_{500\ m\mu}/E_{280\ m\mu}$ = 0·017). Over the wavelength range investigated (260–740 mμ) these were the only two bands observed in the absorption spectra of the final preparations. Fig. 3 compares the absorption, in the visible region of the spectrum, of one of the initial preparations with two of the final preparations. In the initial preparations the presence of the pink compound was obscured by coloured impurities and the absorption spectra showed only slight inflexions in the region of 500 mμ. The 500 mμ band was shown by all the final preparations and the results of Figs. 2 and 3 suggested that its intensity was related to the concentration of amine oxidase.

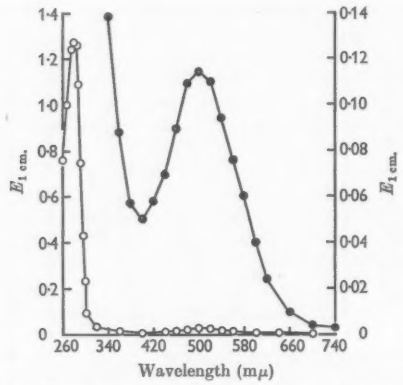

Fig. 2. Absorption spectra of one of the final amine-oxidase preparations. ●, Preparation 4 (Table 1) containing 254 000 units/ml. (right-hand ordinate); ○, the same preparation diluted 1:5 with water (left-hand ordinate).

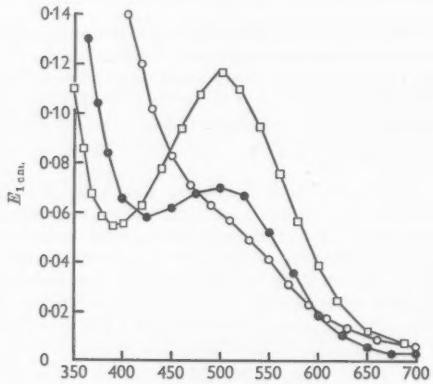

Fig. 3. Absorption spectra of initial and final preparations of the amine oxidase. ○, Initial preparation 3 (Table 1) containing 46 000 units/ml.; ●, final preparation 3 containing 153 000 units/ml.; □, final preparation 6 containing 250 000 units/ml.

Reaction with dithionite. The pink colour of the enzyme preparations was discharged by sodium dithionite and restored by bubbling O_2 through the solution. Fig. 4 shows the corresponding changes in the absorption spectrum of the enzyme solution over the wavelength range 400–700 mμ during these reactions. The absorption at 500 mμ decreased rapidly during the first few minutes after addition of the dithionite and thereafter more slowly and showed little further change after 45 min. The return of the colour when O_2 was subsequently bubbled through the solution was accompanied by an almost complete restoration of the original absorption spectrum.

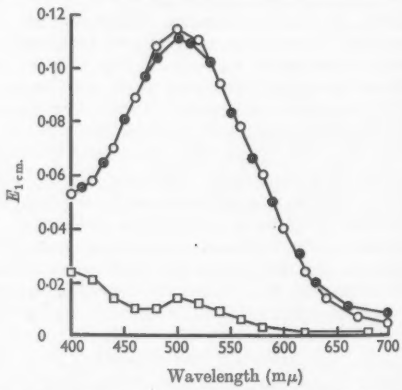

Fig. 4. Reduction of a purified preparation of the amine oxidase with $Na_2S_2O_4$ and re-oxidation with O_2. The amine oxidase was a solution, containing 0·775 mg. of N/ml., of a preparation of specific activity 328×10^3 in 200 mM-phosphate buffer, pH 7. Absorption spectra: ○, before addition of $Na_2S_2O_4$; □, 1 hr. after the addition of 1 mg. of $Na_2S_2O_4$ to 3 ml. of the enzyme solution; ●, after subsequent oxygenation, for 10 min., of the mixture of enzyme and $Na_2S_2O_4$.

Reaction with 1:4-*diaminobutane*. A mixture of amine-oxidase preparation and catalase was placed in the cell and the 1:4-diaminobutane was placed in the Thunberg-type hollow stopper. The assembly was then evacuated. Fig. 5 shows the changes in the absorption spectrum of the mixture. The pink colour changed to yellow on addition of the 1:4-diaminobutane and the band at 500 mμ was replaced by bands with maxima at 465, 435 and 350 mμ. The change from yellow to pink on oxygenation was accompanied by the disappearance of the bands at 465, 435 and 350 mμ and the reappearance, at its original intensity, of the band at 500 mμ.

Fig. 6 shows the absorption spectra of pyridoxal phosphate and of mixtures of pyridoxal phosphate with 1:4-diaminobutane at pH 7. The absorption spectrum of pyridoxal phosphate at pH 7 shows bands with maxima at 330 and 388 mμ. Addition of 1:4-diaminobutane intensified the yellowness of the solution. This change was correlated with a shift of the 388 mμ band to longer wavelengths; the 330 mμ band was reduced in intensity and a new band appeared with maximum absorption at about 275 mμ. These changes, which may be attributed to the formation of the Schiff's base (Matsuo, 1957), were pronounced only when the concentration of

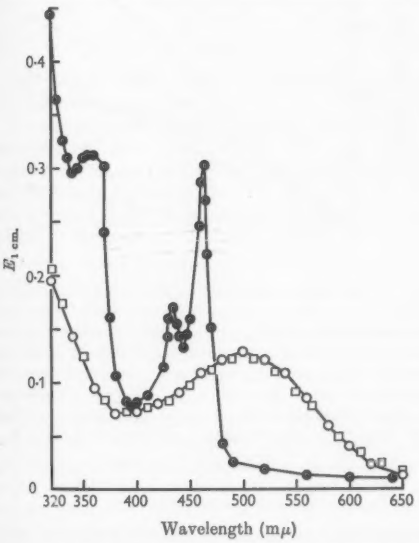

Fig. 5. Reaction of a purified amine-oxidase preparation with 1:4-diaminobutane. The amine oxidase was a solution (2·5 ml.), containing 0·900 mg. of N/ml., of a preparation of specific activity 278×10^3 in 200 mM-phosphate buffer, pH 7. Catalase (25 μg.) was added to this solution. Absorption spectra: ○, before addition of 1:4-diaminobutane; ●, after addition, under anaerobic conditions, of 0·1 ml. of 10 mM-1:4-diaminobutane to 2·5 ml. of the enzyme solution; □, after subsequent oxygenation of the mixture of enzyme and 1:4-diaminobutane.

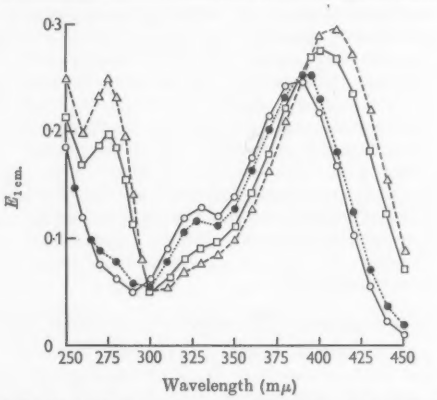

Fig. 6. Absorption spectra of pyridoxal phosphate and of mixtures of pyridoxal phosphate and 1:4-diaminobutane. Reaction mixtures contained 0·05 mM-pyridoxal phosphate, with and without 1:4-diaminobutane, in 100 mM-phosphate buffer, pH 7. The mixtures were allowed to stand for 1 hr. before the absorption spectra were determined. ○, Without 1:4-diaminobutane; ●, 0·33 mM-1:4-diaminobutane; □, 3·3 mM-1:4-diaminobutane; △, 10 mM-1:4-diaminobutane.

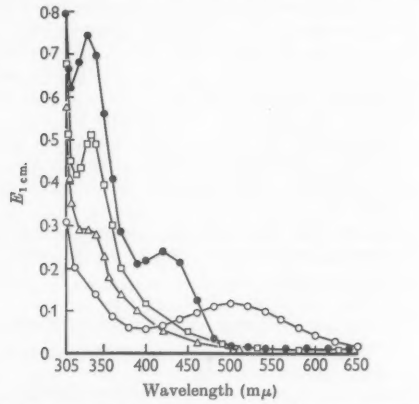

Fig. 7. Reaction of a purified amine-oxidase preparation with hydrazine. The amine oxidase was a solution, containing 0·900 mg. of N/ml., of a preparation of specific activity 278×10^3 in 200 mM-phosphate buffer, pH 7. Absorption spectra were determined before (○) addition of hydrazine sulphate and 10 min. (●), 3 hr. (□) and 6 hr. (△) after addition of 0·1 ml. of 10 mM-hydrazine sulphate to 3 ml. of the enzyme solution.

1:4-diaminobutane was very high in relation to that of the pyridoxal phosphate.

Reaction with hydrazine. Fig. 7 shows the changes in the absorption spectrum of the final enzyme preparation produced by addition of hydrazine sulphate. When the hydrazine was added, the mixture changed from pink to yellow and the band at 500 mμ was replaced by bands at 420 and 330 mμ. On standing in air at room temperature the yellow slowly faded but the mixture did not again become pink. The decrease in the intensity of the yellow was correlated with a decrease in the intensity of the band at 420 mμ, which, after 3 hr., was no longer detectable in the absorption spectrum of the solution. The band at 330 mμ also decreased in intensity, though more slowly, on standing; it was still apparent in the absorption spectrum after 6 hr.

Investigations on the nature of the essential metal

Metal content. Spectrographic analysis of one of the final preparations (1, Table 1) showed that manganese and copper were the trace elements present in highest concentrations. Zinc and iron were present in lower, but possibly significant, amounts. Table 2 shows the results of colorimetric analyses of the copper and manganese contents of the initial and final preparations. The initial preparations contained more manganese than copper but during purification the copper content increased whereas the manganese content decreased. In the final preparations (4–6), the copper content rose to 0·08–0·09 % while the manganese content fell to about 0·01 %. These results were confirmed by a spectrographic analysis of preparation 6, which gave a copper content of 0·085 % and a manganese content of 0·006 %. The spectrographic analysis also showed that, except for zinc, the concentration of which was estimated to be $<0·06$ %, other trace elements, including iron and molybdenum, were present at concentrations $<0·004$ %.

Inactivation by dialysis against diethyldithiocarbamate and reactivation by Cu^{2+} *ions.* The potassium phosphate buffer and 1:4-diaminobutane used in these experiments were freed from traces of copper by extraction with a solution of diphenylthiocarbazone (dithizone) in CCl_4, as described by Hewitt (1952), and then freed from traces of dithizone by extraction with CCl_4.

A sample (4 ml.) of one of the final preparations (5) was dialysed, at 0–5°, in Visking cellulose tubing against 40 ml. of 10 mM-sodium diethyldithiocarbamate in 200 mM-phosphate buffer, pH 7. The colour of the enzyme preparation inside the sac changed from pink to golden yellow within 15 min., and a yellow precipitate was slowly deposited, leaving the supernatant solution pink. After 24 hr. the precipitate (A) and the supernatant solution (B) were separated by centrifuging.

The precipitate (A) was dissolved in 10 ml. of CCl_4. The absorption spectrum of the resulting yellow solution resembled solutions of the copper

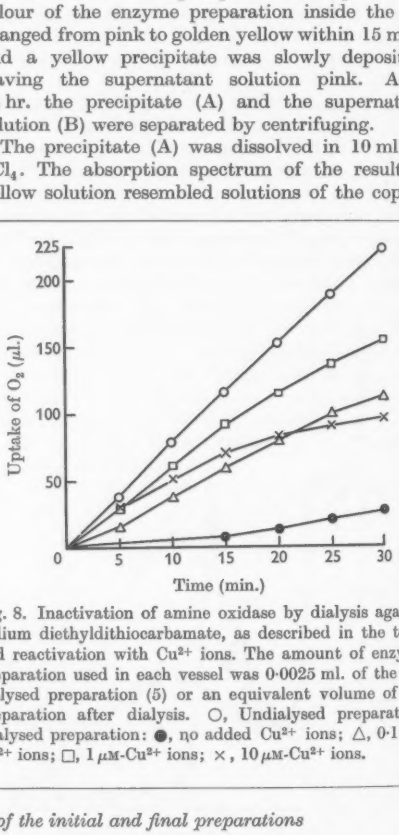

Fig. 8. Inactivation of amine oxidase by dialysis against sodium diethyldithiocarbamate, as described in the text, and reactivation with Cu^{2+} ions. The amount of enzyme preparation used in each vessel was 0·0025 ml. of the undialysed preparation (5) or an equivalent volume of the preparation after dialysis. ○, Undialysed preparation. Dialysed preparation: ●, no added Cu^{2+} ions; △, 0·1 μM-Cu^{2+} ions; □, 1 μM-Cu^{2+} ions; ×, 10 μM-Cu^{2+} ions.

Table 2. *Copper and manganese contents of the initial and final preparations*

Results are based on the protein contents of the preparations, which were calculated from the N contents by using the factor of 6·25.

	Initial preparation			Final preparation		
Preparation no.	10^{-3} × Specific activity	Cu (%)	Mn (%)	10^{-3} × Specific activity	Cu (%)	Mn (%)
1	89	0·040	0·069	191	0·063	0·059
2	98	0·046	0·074	235	0·062	0·049
3	105	0·045	0·066	219	0·057	0·038
4	108	0·053	0·067	328	0·082	<0·01
5	75	0·031	0·072	277	0·079	<0·01
6	97	0·042	0·063	278	0·092	0·014

diethyldithiocarbamate complex in CCl_4, and showed a band with maximum absorption at 436 mμ. Assuming the extinction at 436 mμ was entirely due to the copper complex it was calculated that the solution contained 12·5 μg. of copper; the copper content of the sample of enzyme used in the experiment was 13·4 μg.

The supernatant solution (B) was dialysed for 16 hr. at 0–5° against 200 ml. of 10 mM-phosphate buffer, pH 7. Fig. 8 shows that the dialysed preparation retained little of the activity of the original preparation; most of the lost activity was restored by the addition of Cu^{2+} ions (as $CuSO_4$, $5H_2O$). Maximum reactivation was given by Cu^{2+} ions at a concentration of 1 μM. Copper ions at concentrations of 0·1–10 μM did not increase the activity of the original preparation but at 10 μM produced an inhibition which increased during the course of the oxidation. With this concentration of Cu^{2+} ions an inhibiting effect was also observed with the dialysed enzyme, after an initial activation (Fig. 8). Zinc ions (added as $ZnSO_4,7H_2O$) at concentrations of 0·1–10 μM had no activating effect on the dialysed preparation.

DISCUSSION

The purest preparations of pea-seedling amine oxidase hitherto reported were made by Uspenskaia & Goryachenkova (1958) by electrophoretic fractionation of initial preparations obtained by the method of Mann (1955). The combined methods gave a 550-fold purification of the enzyme but the final preparations were unstable and lost all their activity within 24 hr. The method now described, involving chromatographic fractionation on hydroxyapatite and DEAE cellulose, yields purer and more stable preparations.

These preparations of the plant enzyme have activities which are much higher than those so far reported for the purified preparations of the related animal diamine oxidase, though exact comparison is rarely possible because different methods are used to express the specific activities. The highly purified preparations of the animal enzyme obtained by Tabor (1951) had a specific activity (enzyme units/mg. of protein), calculated from the rate of oxidation of 1:4-diaminobutane at 37·5°, of 670. The preparations of the plant enzyme obtained by the present method had an average specific activity (enzyme units/mg. of nitrogen), calculated from the same reaction at 28°, of 294×10^3. Taking into account the fact that the plant enzyme catalyses the oxidation of 1:4-diaminobutane about twice as rapidly at 37·5° as at 28°, and assuming a nitrogen content of 16 %, it may be calculated that the plant preparations were about 140 times as active as the animal preparations. The purest preparations of animal diamine oxidase so far obtained appear to be those of Uspenskaia & Goryachenkova (1958), which were claimed to be about three times as active as those of Tabor (1951).

During preliminary investigations of the substrate specificity of the enzyme it was found that the oxidation of di-β-phenylethylamine was not catalysed either by the final preparations or by pea-seedling extracts. The oxidation observed by Kenten & Mann (1952) may thus have been that of an impurity in the sample of di-β-phenylethylamine used.

The results of the spectrophotometric investigations of the final enzyme preparations suggest that the enzyme itself is pink; the intensity of the band with a maximum at 500 mμ is related to the concentration of enzyme in these preparations. The pink compound is easily reduced by sodium dithionite and the reduced form is auto-oxidizable; it may function as a hydrogen carrier in the enzyme-catalysed reactions.

The fact that the pink colour of the preparations is discharged by 1:4-diaminobutane under anerobic conditions, and is restored by subsequent oxygenation, could mean that the pink compound is involved in the catalytic activity of the enzyme and that it may undergo a cycle of reduction and re-oxidation during such activity. The plant amine oxidase belongs to the group of amine oxidases which are inhibited by hydrazine, so the fact that the colour is discharged by hydrazine provides further evidence that the enzyme is pink. This reaction suggests that the pink compound may be a carbonyl compound; the primary reaction product is yellow and absorbs maximally at 420 mμ, which indicates that an aromatic carbonyl compound is involved. Some of the Schiff's bases of pyridoxal phosphate, which are yellow and absorb maximally at 415 mμ, are unstable and gradually change to colourless forms absorbing maximally at 330–335 mμ or 278–285 mμ (Christensen, 1958). The resemblance between the behaviour of these Schiff's bases and that of the product of the reaction of hydrazine with the enzyme preparations supports the suggestion that an aromatic carbonyl compound is involved in this reaction. If this carbonyl compound is pyridoxal phosphate, it is not split from the enzyme during the reaction with hydrazine, because the hydrazone of pyridoxal phosphate is stable in phosphate buffer, pH 7, and absorbs maximally at 284 and 320 mμ (Curry & Bulen, 1960). Assuming that the catalytic activity of the enzyme depends on the oxidation and reduction of an aromatic carbonyl compound present in its prosthetic group, the spectrum of the mixture of the enzyme preparation and 1:4-diaminobutane, under anaerobic conditions, may

be regarded as that of a Schiff's base formed between the reduced enzyme and the substrate. The fact that the absorption spectrum of the enzyme was changed greatly by low concentrations of 1:4-diaminobutane suggests that this enzyme–substrate compound is largely undissociated. The spectrophotometric investigations of the Schiff's base formed by 1:4-diaminobutane with free pyridoxal phosphate suggests that this compound is largely dissociated at pH 7.

The absorption spectra of the final enzyme preparations are not typical of an enzyme containing flavinadenine dinucleotide or pyridoxal phosphate in its prosthetic group. Enzymes which contain flavinadenine dinucleotide in their prosthetic groups show the characteristic absorption bands of this compound, whose maxima, at 375 and 450 mμ, are usually only shifted some 5–15 mμ, generally towards longer wavelengths, by combination with the apoenzymes. Thus the absorption maxima are at 377 and 455 mμ with glucose oxidase (notatin) (Keilin & Hartree, 1948) and at 389 and 465 mμ with the L-amino acid oxidase of mocassin venom (Singer & Kearney, 1950). In the absorption spectra so far described of enzymes containing pyridoxal phosphate, the position of the absorption band attributed to this compound varies with pH between about 330 and 430 mμ (e.g. glutamic acid decarboxylase; Shukuya & Schwert, 1960). When such enzymes are treated with 0·1N-sodium hydroxide, the pyridoxal phosphate is split off and the absorption spectrum then shows the band with maximum at 388 mμ characteristic of pyridoxal phosphate in neutral or alkaline solution. The pinkness of the amine-oxidase preparations is little affected by changes of pH over the range pH 5–10. The colour is discharged by 0·1N-sodium hydroxide but no evidence has so far been obtained from the absorption spectra that this change is correlated with the formation of free pyridoxal phosphate.

The copper content of the final preparations and the increase in copper content during the final purification indicate that the amine oxidase may be a copper-containing enzyme. This suggestion is supported by the results of the preliminary attempts to prepare the copper-free enzyme, by precipitation of the copper with diethyldithiocarbamate, and to demonstrate its activation by Cu^{2+} ions. The magnitude of this effect of Cu^{2+} ions has varied widely in different experiments, and conditions have not yet been established in which complete inactivation and reactivation of the enzyme always occurs. Further work is necessary to establish such conditions and to prove that the observed restoration of activity by Cu^{2+} ions is a true reactivation and not a de-inhibition, and that this reactivation is brought about specifically by Cu^{2+} ions. The copper is apparently present in the enzyme in the cupric form; the possibility that the catalytic activity involves a valency change of the copper has not yet been investigated. Most complexes of cupric copper are blue or green but the only absorption band shown by the enzyme preparations in the visible part of the spectrum is that from the pink compound. It is therefore suggested that the copper occurs as a complex with the carbonyl compound and that this complex forms the prosthetic group of the enzyme.

SUMMARY

1. The amine oxidase of pea-seedling extracts was further purified by column chromatography on hydroxyapatite and diethylaminoethyl cellulose. This procedure, together with the preliminary fractionation previously described, gave an 880-fold purification.

2. Spectrophotometric investigations were made of the purified enzyme and of its reactions with sodium dithionite, 1:4-diaminobutane and hydrazine.

3. The final preparations were pink solutions with maximum absorption at about 500 mμ; this was the only band detected in the visible part of the spectrum.

4. The colour was discharged by sodium dithionite and restored by oxygenation.

5. The enzyme solution changed from pink to yellow when 1:4-diaminobutane was added in anaerobic conditions. The pink was restored by oxygenation of the mixture.

6. The colour of the enzyme solution changed from pink to yellow when hydrazine was added. On standing in air the yellow slowly faded but the mixture did not again become pink.

7. The copper content of the enzyme preparations increased during purification to 0·08–0·09%. The copper was removed by precipitation with sodium diethyldithiocarbamate. The supernatant solution, after dialysis to remove sodium diethyldithiocarbamate, retained little of the activity of the original preparation. Most of the lost activity was restored by the addition to the dialysed solution of Cu^{2+} ions.

8. It is suggested that the pink colour is due to a carbonyl compound and that a copper complex of this compound forms the prosthetic group of the enzyme and undergoes a cycle of reduction and reoxidation during the catalytic activity.

I thank Dr H. H. Le Riche for the spectrographic analyses of the purified enzyme preparations and Unicam Instruments Ltd. for the gifts of the Thunberg-type assemblies used in the spectrophotometry.

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The Liver-Lipid Constituents of Male and Female Rats

1. EFFECTS OF THE FAT-DEFICIENCY SYNDROME

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Green, Horner, Lowe & Morton (1957), studying the effect of feeding cholesterol to rats, noted a sex difference in that the treatment decreased liver vitamin A reserves in males but not in females.

It is well established that male rats have a much higher requirement for linoleic acid than females (see, for example, Greenberg, Calbert, Savage & & Deuel, 1950). Linoleate is the predominant ester of cholesterol in the plasma of healthy rats (Klein, 1957). Peifer & Holman (1955) and Holman & Peifer (1960) have shown that dietary cholesterol accelerates the onset of fat deficiency.

On such evidence, Green *et al.* (1957) suggested that the sex difference which they had observed might be no less closely linked with the intake of polyunsaturated fatty acids than with that of cholesterol.

The experiments to be described were planned to reveal any sex difference in respect of liver vitamin A stored by fat-deficient rats. The paper immediately following (Horner & Morton, 1961) is concerned with similar experiments in which fat-deficient rats were fed with cholesterol.

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Liver lipids other than vitamin A were also determined. The cholesteryl esters, triglycerides and phospholipids were separated on silicic acid columns, and the polyunsaturated acids from these fractions were analysed by alkali-isomerization. These procedures were first applied by Mead & Fillerup (1954) to the study of rat plasma lipids. They have since been used by several workers to observe the incorporation into the esters of liver and plasma of the polyunsaturated fatty acids supplied by various edible fats.

In the present work the emphasis is on differences between the sexes and between fat-deficient animals and controls given the minimum amount of linoleic acid believed to be essential for good growth.

EXPERIMENTAL

Animals and diets. Hooded rats were fed on the following basal fat-free diet from weaning: sucrose, 73%; casein (Genatosan), 21%; salts (Hubbell, Mendel & Wakeman, 1937), 4%; Monotype paper punchings, 2%. The vitamin mixture (Table 1) was added (3 g./kg. of diet).

Procedure. Nine male rats and nine females selected from four litters born over a period of 5 days were given the fat-free diet from weaning. Three days after the youngest

animals had been weaned four experimental groups were formed. In group B, five males received the basal diet, and in group A, four males received the basal diet plus 100 mg. of linoleic acid per day. In group D, four females received the basal diet, and in group C, five females received the basal diet plus 25 mg. of linoleic acid per day.

The diets were fed *ad lib.* and water was freely provided. Linoleic acid was given in the form of the calculated volume of a tested cottonseed oil containing 54% of linoleic acid. The oil was added daily by pipette to a portion of the food of each individual animal. This was eaten promptly.

The male rats were killed after 190 days and the females after 200 days.

Vitamin A dosage. Every rat received eleven equal doses each of 800 i.u. of vitamin A given at weekly intervals from the start of the experiment. A known weight of synthetic vitamin A acetate (F. Hoffmann–La Roche and Co. Ltd.) was dissolved in a mixture of 95% ethanol (1 vol.) and glycerol (9 vol.). The standard dose was given orally by pipette in a volume of 0·2 ml. The dose also contained 2 mg. of DL-α-tocopheryl acetate.

Analysis of livers. The rats were killed with $CHCl_3$ vapour. Livers were washed, roughly dried on filter paper and weighed. Each liver was ground with anhydrous Na_2SO_4 and silver sand. The mixture was exhaustively extracted by boiling briefly with five 100 ml. portions of ether, and the bulked extracts were dried under N_2 on a water bath and dried under suction to constant weight. One-half of each sample of lipid was saponified for spectrophotometric estimation of vitamin A on the unsaponifiable fraction (Cama, Collins & Morton, 1951). The remaining portions of lipid were bulked within each experimental group and chromatographed.

Chromatography on silicic acid. The procedure was essentially that of Fillerup & Mead (1953); hydrocarbons, cholesteryl esters, triglycerides and free cholesterol were eluted with light petroleum (b.p. 40–60°) and diethyl ether in light-petroleum mixtures containing 2, 4 and 10% of ether (v/v) respectively. Phospholipids were eluted with methanol–ether (3:1, v/v) followed by absolute methanol.

Moist silicic acid (British Drug Houses Ltd.) was heated at 120° for 24 hr. and then ground mechanically to a fine powder. This was stirred three times with successive portions of warm methanol, the first of which removed a yellow contaminant. Particles of silicic acid which did not settle in methanol were removed by decantation. After three further washings with warm redistilled ether the material was spread on filter paper to dry and later was again heated at 120° for 24 hr., cooled in a desiccator and stored in a tightly stoppered bottle. Most of the material was of particle size in the range 40–160 standard mesh.

Samples of lipid, each weighing 500–700 mg., were chromatographed on 30 g. of prepared silicic acid in columns of internal diameter 2·5 cm. with glass centre-rods of diameter 0·5 cm. About 200 ml. of solvent above the top of the adsorbent gave a head sufficient to maintain a good rate of flow.

The final fraction eluted with methanol usually contained silicic acid. The methanol was distilled off under reduced pressure, and the phospholipids were redissolved in ether and decanted from the insoluble residue.

Preparation of fatty acids. Free fatty acids for alkali-isomerization were obtained by the following methods from the various fractions.

(*a*) Cholesteryl esters were saponified on a boiling-water bath with N-KOH in ethanol for 1 hr. with frequent vigorous shaking. The mixture was acidified with conc. HCl and extracted with ether. The extract was freed from solvent, dried and dissolved in light petroleum and then chromatographed on a short column of $MgCO_3$. A failure by light petroleum to elute any material was accepted as evidence of complete hydrolysis. Cholesterol was eluted with ether. The column was then dissolved in warm acidified ethanol, and the fatty acids were extracted with ether.

(*b*) Triglycerides and phospholipids were saponified with N-KOH in ethanol for 30 min. and 1 hr. respectively. The mixtures were acidified and extracted with ether. The product from phospholipids was expected to contain a little unsaponified material (see Lovern, 1955).

Alkali-isomerization. Samples of fatty acids weighing 1–10 mg. were alkali-isomerized by the micro-method of Holman & Hayes (1957). Analyses were made in triplicate and usually agreed well. (In a few cases turbidity raised extinction values considerably so that the results had to be rejected.)

Table 1. *Vitamin mixture*

	%	mg./100 g. of ration
Thiamine hydrochloride	0·167	0·5
Riboflavin	0·334	1·0
Pyridoxine hydrochloride	0·135	0·4
Calcium pantothenate	1·667	5·0
Nicotinic acid	0·334	1·0
Choline chloride	64·652	200·0
*meso*Inositol	32·326	100·0
p-Aminobenzoic acid	0·334	1·0
Folic acid	0·033	0·1
Biotin	0·003	0·01
Menaphthone	0·015	0·05

RESULTS

Body weights and liver weights together with the liver-lipid analyses are given in Table 2.

Growth and deficiency symptoms. Deficient males and females ceased to increase in weight after 160 and 170 days respectively. Weights remained steady until the animals were killed after 190 and 200 days. All the rats showed the classical external signs of fat-deficiency (e.g. Burr & Burr, 1929) as well as the brown pigmentation of originally black hair reported by Basnayake & Sinclair (1955). Various symptoms and, in particular, scaly tails were more pronounced in the males than in the females. The control groups (both sexes) were still increasing in weight and seemed quite healthy at the end of the experimental periods.

Liver vitamin A. The mean total liver vitamin A value for deficient males was 5·25% higher than that of the control males but the difference lacked statistical significance ($t = 0{\cdot}398$, $P > 0{\cdot}5$). The value for deficient females was 9·9% lower than that of their controls, but again the difference was not significant ($t = 0{\cdot}836$, $P > 0{\cdot}4 < 0{\cdot}5$).

Liver lipids. Data are presented for all the alkali-conjugatable polyunsaturated fatty acids, but, as knowledge of the metabolism of pentaenoic and hexaenoic acids is still fragmentary and no consistent changes were noted, the results for these acids will not be discussed.

The concentration of lipid in the livers of deficient males was 1·52 times that of the control males, whereas in the deficient females the corresponding value was very little higher (1·08 times) than the level shown by the control females. Cholesteryl ester concentrations were higher both in deficient males (3·36 times the control concentration) and deficient females (2·36 times the control level). Triglyceride concentrations also were higher in deficient animals, being 3·47 times the control group concentration in males, but only 1·15 times in females. The concentrations of other constituents of the lipid material remained practically unchanged in fat deficiency.

A comparison of the two control groups shows that cholesteryl ester concentration in males was 1·31 times that of the females but the male triglyceride concentration was approximately half that of the female group.

There were marked changes in the conjugatable fatty acids in the livers of deficient animals. In males the total weights of dienoic and tetraenoic acids fell to 0·35 and 0·29 respectively of the control values, and trienoic acid rose by a factor of 2·47 times. In deficient females total dienoic and tetraenoic acids fell to 0·44 and 0·27 respectively of the control values, and trienoic acid rose by a factor of 1·71.

In control females the total weight of trienoic acid was higher (by a factor of 1·34) and the total

Table 2. *Body weights and liver weights, liver vitamin A and the weights of the various lipid fractions and their constituent fatty acids in the livers of fat-deficient male rats (group B) and their healthy controls (group A) and in fat-deficient female rats (group D) and their controls (group C)*

Control males were given 100 mg., and control females 25 mg., of linoleic acid per day.

Group	A	B	C	D
Mean final body wt. (g.)	288	167	196	133
Mean liver wt. (g.)	9·81	7·06	7·33	6·06
Mean total liver vitamin A (i.u.)	704 (±56)*	741 (±69)	996 (±92)	897 (±64)
Mean total liver lipid (mg.)	243	266	222	193
Mean wt. (mg.)/liver of:				
Phospholipids	134·0	96·0	106·2	84·0
Triglycerides	47·3	118·0	74·3	68·9
Cholesteryl esters	8·0	19·2	4·7	8·8
Free cholesterol	31·3	22·5	20·3	18·7
Hydrocarbons	3·6	3·1	1·7	1·7
Mean wt. (mg.)/liver of:	Phospholipid fatty acids			
Dienoic	4·40	1·11	3·06	0·86
Trienoic	2·20	5·63	3·07	5·38
Tetraenoic	6·16	1·67	4·91	1·33
Pentaenoic	2·18	0·32	2·22	0·25
Hexaenoic	0·95	0·03	1·26	0·36
Total polyunsaturated acids	15·89	8·76	14·52	8·18
Monoenoic and saturated	40·39	34·92	42·38	23·16
Mean wt. (mg.)/liver of:	Triglyceride fatty acids			
Dienoic	1·68	0·91	1·05	0·86
Trienoic	0·32	0·71	0·39	0·54
Tetraenoic	0·21	0·13	0·23	0·09
Pentaenoic	0·07	0·06	0·08	0·05
Hexaenoic	0·14	0·14	0·24	0·12
Total polyunsaturated acids	2·42	1·95	1·99	1·66
Monoenoic and saturated	39·39	104·25	63·25	58·17
Mean wt. (mg.)/liver of:	Cholesteryl ester fatty acids			
Dienoic	0·23	0·21	0·10	0·08
Trienoic	0·11	0·16	0·06	0·08
Tetraenoic	0·08	0·06	0·11	0·02
Pentaenoic	0·01	0·01	0·02	0·004
Hexaenoic	0·04	0·02	0·04	0·02
Total polyunsaturated acids	0·47	0·46	0·33	0·20
Monoenoic and saturated	4·01	7·87	1·76	3·06

* Standard deviation of the mean.

dienoic acid was lower (by a factor of 0·67) than in male controls. These values probably reflect the differences in cottonseed oil dosage; males received four times the dose given to females.

Changes in polyunsaturated fatty acid composition due to fat deficiency were most marked in the phospholipids. In both deficient groups the weight of phospholipid-bound tetraenoic acid was only 0·27 that of the control value. In deficient animals phospholipid trienoate was 2·56 times the control value in males and 1·75 times in females. Phospholipid dienoate in deficient males was 0·25 times the control level and in deficient females 0·28 times the level for control females.

Falls in dienoic and tetraenoic acids and rises in trienoic acid levels were also evident in the triglycerides of deficient animals. In terms of absolute weights of acids involved the changes were much less marked than those of the phospholipid fatty acids, and on a concentration basis also they were less striking. Thus in deficient males the weights of triglyceride dienoate and tetraenoate fell to 0·54 and 0·62 of their control values, whereas triene rose by a factor of 2·22. In deficient females the corresponding factors were 0·82, 0·39 and 1·38.

Changes in polyunsaturated fatty acid composition caused by fat deficiency were least evident in the cholesteryl esters. Differences from normal followed the same pattern as in other lipid groups and were slight, with one exception; tetraenoic acid in females fell to 0·16 times the control value; in males the corresponding change was a fall to 0·75 times the control level.

DISCUSSION

Liver vitamin A stores were not significantly affected by fat deficiency in either male or female rats. The amount of vitamin A stored in the liver depends both on the efficiency of absorption and of storage and on the rate at which it is subsequently utilized. It is still possible that control rats may initially accumulate larger vitamin A reserves than the fat-deficient animals. The controls are, however, appreciably larger animals and they may draw more rapidly on the reserves and thus decrease their stores to levels similar to those of their deficient counterparts. Experiments to measure vitamin A utilization have been performed and will be reported separately.

Deficiency of fat causes a rise in liver-cholesterol concentrations. This effect has been reported previously by Deuel, Alfin-Slater, Wells, Kryder & Aftergood (1955). The present work shows that the increase is entirely in respect of esterified cholesterol and is more pronounced in males than in females.

Klein (1958) compared the fatty acids of the liver cholesteryl esters under various conditions of linoleate intake and concluded that the concentrations of cholesteryl esters reflected the amount of linoleate fed, being high with none, low with some and high again with more. His results cannot be closely compared with ours, for daily linoleic acid intakes of groups receiving fat would increase throughout the experiment as more food was eaten by the growing rats (fat was an integral part of the diet). However, Klein (1958) gave data for one group of male rats on a fat-free diet. If weights of cholesteryl ester fatty acids are expressed as mg./100 g. of liver, Klein's values are: dienoic, 3·6 (3·0); trienoic, 1·2 (2·3); tetraenoic, 0·1 (0·8); pentaenoic, 0·2 (0·1). Our own values for group B are given in parentheses; these rats were almost certainly more severely fat-deficient than Klein's corresponding group. The lower dienoic and higher trienoic levels support this, but the considerably higher tetraenoic value is anomalous.

Klein (1958) found a direct relationship between the dietary level of linoleic acid and its concentration in the liver cholesteryl esters. The data for our control groups A and C fit very well the straight line which correlates the logarithms of Klein's variables. (The mean individual food intakes of rats in groups A and C were assessed at 15 g. and 12 g. per day respectively.)

Table 2 brings to light many marked differences between liver-lipid levels in males and females. The rise in triglyceride concentration in deficient males was the most striking change of all, whereas the corresponding effect of fat deficiency in females was very small.

The falls in total liver tetraenoic acid were very similar in deficient males and females relative to their respective controls, but decreases in dienoic and increases in trienoic acids were more marked in males.

Phospholipid concentrations were not altered by fat deficiency, but the changes in the pattern of polyunsaturated fatty acids were very marked and followed the pattern already described for liver fatty acids as a whole. This was to be expected as the phospholipids were much richer in polyunsaturated acids than were the other lipids.

Reiser, Gibson, Carr & Lamp (1951) found that the rise in trienoic acid in egg-yolk fat from fat-deficient hens was five times as great in the phospholipids as in the neutral fat.

In triglycerides and cholesteryl esters also, rises in trienoic and falls in dienoic acid concentrations were more marked in males than in females. The qualitatively important difference from the overall pattern of changes in the phospholipids and liver lipids as a whole was that the falls in triglyceride and cholesteryl ester tetraenoic acid were very much greater in females than in males.

The extra triglycerides and cholesteryl esters which accumulated in fat-deficient animals were not esterified with polyunsaturated acids other than trienoic acid.

Values for the concentrations and ratios of dienoic, trienoic and tetraenoic acids in each lipid fraction are tabulated in the following paper (Horner & Morton, 1961), which compares the effects of fat deficiency itself with effects due to the addition of cholesterol to a fat-free diet.

Analysis by alkali-isomerization throws little light on the complex changes in trienoic acid levels which occurred in fat deficiency. The triene which accumulates in this condition is eicosa-5:8:11-trienoic acid (Mead & Slaton, 1956), which the animal forms from oleic acid (Fulco & Mead, 1959). In our experiments the picture is complicated by the fact that the cottonseed oil given to controls contained some linolenic acid (0·74%). Therefore male and female controls received 1·36 and 0·34 mg. of linolenic acid per day respectively.

In the present work most of the changes from normal in liver-lipid constitution, i.e. accumulation of cholesteryl esters and triglycerides, a decrease in dienoic acid and an increase in trienoic acid, were more marked in males than in females.

Thus, as with the effect of cholesterol on liver vitamin A (Green *et al.* 1957), the female rat is more resistant than the male to nutritional hazards which disturb lipid metabolism.

There are, however, instances in which the converse holds. The accumulation of liver lipid caused by feeding a choline-free diet is more marked in female rats than in males (Deuel & Hallman, 1941). Females also show the greater 'exogenous ketonuria' when fed ketogenic acids after a period of inanition (Butts & Deuel, 1933). They are also less resistant to the 'endogenous ketonuria' which results from feeding a high-fat, low-protein, choline-free diet (Deuel, Hallman & Murray, 1937). Artom (1959) has reported that large injected doses of DL- or L-ethionine given to female rats caused some increase in liver-triglyceride concentration and markedly impaired the oxidation of fatty acids by liver homogenates. These effects were not seen in males.

SUMMARY

1. The liver vitamin A stores of male and female fat-deficient rats did not differ significantly from those of their controls which received linoleic acid, administered as cottonseed oil.

2. Fat-deficient rats had higher liver-lipid concentrations than their controls, owing to increases in cholesteryl esters and triglycerides. Both effects were more marked in males than in females.

3. Control males, which received 100 mg. of linoleic acid per day, had considerably higher cholesteryl ester concentrations and lower triglyceride concentrations than control females which received 25 mg. of linoleic acid per day.

4. The total weights of dienoic and tetraenoic fatty acids fell, and the weight of trienoic acid rose, in the livers of fat-deficient rats. Quantitatively these effects were mostly due to changes in the phospholipid fatty acids.

5. Falls in total liver and phospholipid-bound tetraenoic acids were almost equally marked in male and female fat-deficient rats. Losses of dienoic and increases in trienoic acids in the total liver lipid and phospholipids were more marked in males.

6. In liver triglycerides and cholesteryl esters, rises in trienoic and falls in dienoic acid levels due to fat deficiency were more marked in males, but tetraenoic acid levels fell to a greater extent in females.

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The Liver-Lipid Constituents of Male and Female Rats

2. EFFECTS OF THE FAT-DEFICIENCY SYNDROME, AGGRAVATED BY DIETARY CHOLESTEROL

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Feeding cholesterol to rats depresses liver vitamin A concentrations in male rats but not in females (Green, Horner, Lowe & Morton, 1957). The difference between the sexes in this effect of cholesterol suggested that there might be a metabolic link with the essential fatty acids. It has been shown, however, that deficiency of these essential fatty acids did not itself affect liver vitamin A levels in either sex (Morton & Horner, 1961). The experiments reported below were begun at the same time as those just referred to and differed only in that all the animals had 1% of cholesterol added to their basal fat-free diets. Previous work (Peifer & Holman, 1955; Holman & Peifer, 1960) had shown that dietary cholesterol accelerated the onset of fat deficiency.

Our first aim was to find how the cholesterol-supplemented diets influenced liver vitamin A levels. During this investigation interrelationships between essential fatty acids and either endogenous cholesterol (Morton & Horner, 1961) or exogenous cholesterol were examined.

EXPERIMENTAL

Animals and diets. Hooded rats were fed on a basal fat-free diet from weaning. This differed from the diet described by Morton & Horner (1961) in that 1% of crystalline cholesterol (Croda Ltd.) was included at the expense of an equal weight of sucrose.

Procedure. The experimental plan resembled that described for rats that did not receive cholesterol (Morton & Horner, 1961). Ten males and twelve females from six litters delivered within a period of six days were given the basal diet at weaning. The experimental groups were formed when weaning was completed. In group 2 five males received the basal diet. In group 1 five males received the basal diet plus 100 mg. of linoleic acid per day. In group 4 six females received the basal diet. In group 3 six females received the basal diet plus 25 mg. of linoleic acid per day. The diets were fed *ad lib.* and water was freely provided. Linoleic acid was given as cottonseed oil.

The males were killed after 78–81 days. The females were killed between the 138th and 146th days.

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Vitamin A dosage. Each rat received eight equal doses of 800 i.u. of vitamin A given by mouth at weekly intervals from the start of the experiment.

Analysis of livers. The methods described by Morton & Horner (1961) were used. The cholesteryl esters from group 4 were accidentally lost during saponification. The value given in Table 1 for the total fatty acids from the fraction was calculated by assuming that the yield of fatty acids from the esters was 36·7% by weight. This was the mean recovery of fatty acids from twenty other samples of cholesteryl esters.

RESULTS

Body weights and liver weights with the liver-lipid analyses are given in Table 1.

Growth and deficiency symptoms. The deficient males had all ceased to grow (i.e. became stationary in weight) by the 72nd day at an average weight of 107 g. Their controls were still growing and then averaged 175 g. Deficient females ceased to grow by the 110th day, averaging 124 g. in weight. Their controls then weighed 173 g. on average.

All the deficient animals had scaly tails but other lesions were relatively mild. In particular, only the hind paws were scaly. All symptoms were less marked in the females and control animals of both sexes were perfectly healthy in appearance.

Liver vitamin A. The mean liver vitamin A of the deficient males was 15% lower than the value for the controls. This difference was not statistically significant at an acceptable level of probability ($t = 1{\cdot}483$, $P < 0{\cdot}2 > 0{\cdot}1$). On the other hand, the average liver vitamin A content of deficient females was 48% lower than the control value and the difference was significant ($t = 3{\cdot}340$, $P < 0{\cdot}01$).

Liver lipids. The results can be compared with those of earlier experiments (Morton & Horner, 1961) in which rats received no cholesterol. In doing so the terms 'normal' males and 'normal' females refer to control groups A and C respectively in the preceding paper.

Concentrations of lipid in the livers of groups 1 and 2 exceeded the 'normal' value by factors of 2·96 and 2·77 respectively. In females also the 'normal' value was exceeded, by factors of 1·11 in group 3 and 1·24 in group 4. These increases over

Table 1. *Body weights and liver weights, liver vitamin A and the weights of the various lipid fractions and their constituent fatty acids in the livers of fat-deficient male rats (group 2) and their controls (group 1) and in fat-deficient female rats (group 4) and their controls (group 3)*

All animals received a dietary supplement of cholesterol (1% by weight). Control males were given 100 mg., and control females 25 mg. of linoleate per day. N.D. indicates not determined.

Group	1	2	3	4
Mean final body wt. (g.)	184	107	198	124
Mean liver wt. (g.)	6·75	5·33	7·78	6·37
Mean total liver vitamin A (i.u.)	1781 (±113)*	1509 (±145)	975 (±134)	507 (±43)
Mean total liver lipid (mg.)	495	364	254	233
Mean wt. (mg.)/liver of:				
Phospholipids	64·1	63·4	106·2	63·2
Triglycerides	96·0	78·1	88·0	69·5
Cholesteryl esters	253·7	196·0	23·8	70·1
Free cholesterol	23·3	20·8	21·4	18·2
Hydrocarbons	5·8	5·9	4·4	3·4
Mean wt. (mg.)/liver of:	Phospholipid fatty acids			
Dienoic	2·30	0·95	2·17	1·19
Trienoic	0·86	3·99	2·53	5·30
Tetraenoic	1·97	1·00	2·99	1·28
Pentaenoic	0·46	0·21	1·30	0·25
Hexaenoic	0·49	0·48	0·47	0·13
Total polyunsaturated acids	6·08	6·63	9·46	8·15
Monoenoic and saturated	19·61	28·74	33·45	29·61
Mean wt. (mg.)/liver of:	Triglyceride fatty acids			
Dienoic	4·35	0·75	1·31	0·64
Trienoic	0·75	0·79	0·57	0·61
Tetraenoic	0·72	0·13	0·38	0·10
Pentaenoic	0·18	0·05	0·12	0·04
Hexaenoic	0·26	0·21	0·20	0·07
Total polyunsaturated acids	6·26	1·93	2·58	1·46
Monoenoic and saturated	71·37	70·34	74·21	58·39
Mean wt. (mg.)/liver of:	Cholesteryl ester fatty acids			
Dienoic	3·79	0·87	0·23	N.D.
Trienoic	0·47	1·21	0·10	N.D.
Tetraenoic	0·90	0·20	0·13	N.D.
Pentaenoic	0·10	0·05	0·02	N.D.
Hexaenoic	0·16	0·06	0·04	N.D.
Total polyunsaturated acids	5·42	2·39	0·52	25·72†
Monoenoic and saturated	70·19	77·21	7·09	

* Standard deviation of the mean.
† Assumed value for total fatty acids from cholesteryl esters (see text).

'normal' liver-lipid concentrations were due to accumulations of cholesteryl esters and triglycerides, whereas phospholipid levels fell slightly.

The concentrations of cholesteryl esters in control and deficient males exceeded the 'normal' value by 46·4 and 45·6 times respectively. The corresponding values for control and deficient females were 4·94 times and 17·7 times greater respectively than the 'normal' female level.

Triglyceride concentrations in control and deficient males were 2·95 and 3·05 times the 'normal' value, whereas in females they exceeded the 'normal' value by factors of only 1·15 in controls and 1·11 in the deficient animals.

In groups 1 and A the combined weights of dienoic and tetraenoic acids in the livers were 12·76 and 14·03 mg. respectively. Values for the concentrations of di-, tri- and tetra-enoic acids for the experimental groups in the present work and in the preceding paper are given in Table 2.

The distribution of polyunsaturated acids between the three classes of liver ester differed markedly in groups 1 and A. The combined weight of dienoic and tetraenoic acids in the cholesteryl esters of the cholesterol-fed group was 15·1 times as high as in the 'normal' group. (However, the concentrations of these acids as percentages of the total cholesteryl ester acids did not increase. The values for groups 1 and A were 6·2 and 6·9% respectively.)

Table 2. *Concentrations of dienoic, trienoic and tetraenoic acids in liver cholesteryl ester, triglyceride and phospholipid fractions and in the total liver lipid*

Concentrations are expressed as percentages by weight of the total acids recovered from each fraction. The data are for the experimental groups in the present work (groups 1, 2, 3 and 4) and in the experiment described in the preceding paper (Morton & Horner, 1961) (groups A, B, C and D). Lipid class: C.E., cholesteryl ester; T.G., triglyceride; Ph., phospholipid.

Group	Lipid class	Diene	Triene	Tetraene	Triene / Tetraene	Diene / Tetraene
1	C.E.	5·01	0·62	1·19	0·52	4·21
	T.G.	5·60	0·97	0·93	1·04	6·04
	Ph.	8·95	3·35	7·67	0·44	1·17
	Total	5·83	1·16	2·01	0·58	2·91
2	C.E.	1·09	1·52	0·25	5·05	4·35
	T.G.	1·04	1·09	0·18	6·08	5·77
	Ph.	2·69	11·28	2·83	3·99	0·95
	Total	1·37	3·20	0·71	4·50	1·93
3	C.E.	3·02	1·31	1·71	0·77	1·77
	T.G.	1·71	0·74	0·49	1·50	3·45
	Ph.	5·06	5·90	6·97	0·85	0·73
	Total	2·91	2·51	2·75	0·91	1·06
4	C.E.	* —	—	—	—	—
	T.G.	1·07	1·02	0·17	6·10	6·40
	Ph.	3·15	14·04	3·39	4·14	0·93
	Total	≮1·49	≮4·79	≮1·12	—	—
A	C.E.	5·13	2·46	1·79	1·38	2·88
	T.G.	4·02	0·77	0 50	1·52	8·00
	Ph.	7·82	3·91	10·95	0·36	0·71
	Total	6·15	2·56	6·29	0·41	0·98
B	C.E.	2·52	1·92	0·72	2·67	3·50
	T.G.	0·86	0·67	0·14	4·73	6·07
	Ph.	2·54	12·89	3·82	3·37	0·66
	Total	1·41	4·11	1·18	3·49	1·20
C	C.E.	4·79	2·87	5·26	0·55	0·91
	T.G.	1·61	0·60	0·35	1·70	4·57
	Ph.	5·38	5·40	8·71	0·62	0·62
	Total	3·39	2·83	4·23	0·67	0·80
D	C.E.	2·45	2·45	0·61	4·00	4·00
	T.G.	1·44	0·90	0·15	6·00	9·56
	Ph.	2·75	17·17	4·24	4·04	0·65
	Total	1·91	6·35	1·53	4·17	1·25

* This fraction was lost during saponification.

The weight of dienoic and tetraenoic acids in group 1 triglycerides exceeded that in group A 2·7 times. In this case the concentration was higher, being 6·5% in the cholesterol-fed group compared with 4·5% in the 'normal' group.

Increased weights of polyunsaturated acids in the cholesteryl esters and triglycerides accumulated 'at the expense of' the phospholipids. The weights of phospholipid dienoic and tetraenoic acids were 0·52 and 0·32 respectively of group A values.

In cholesterol-fed and 'normal' females, groups 3 and C, the combined weights of dienoic and tetraenoic acids in the livers were 7·21 and 9·46 mg. respectively. The distribution of these acids among the different classes of liver ester differed less strikingly than in the corresponding groups of males, because liver-lipid levels in females were much less influenced by cholesterol feeding.

The weight of dienoic plus tetraenoic acids in the cholesteryl esters of group 3 was 1·71 times the value for group C. The concentrations of these acids as fractions of the total acids esterified with cholesterol were 0·05 in group 3 and 0·1 in group C.

The weight of triglyceride-bound dienoic and tetraenoic acids in group 3 exceeded that of group C by a factor of 1·32, but the fractional concentrations of these acids were almost the same for the two groups.

In group 3 the weight of phospholipid-bound tetraene was 0·65 of that in group C. Phospholipid diene in group 3 was 0·94 of the group C value.

Comparisons may also be drawn solely between groups in the present experiment. In group 2, the total weights of liver dienoic and tetraenoic acids were smaller (0·25 and 0·37 respectively) than the values for the control, group 1. Trienoic acid was

2·88 times higher in the deficient group. The falls in dienoic and tetraenoic acid levels were most marked in the triglycerides and least pronounced in the phospholipids. Conversely, the rise in trienoic acid was greatest in the phospholipids and least in the triglycerides.

The accidental loss of the cholesteryl esters from group 4 prevents a full comparison being made between the groups of females. Changes from the control weights of dienoic and tetraenoic acids were greater in the triglycerides; the rise in triene was more marked in the phospholipids.

DISCUSSION

In males, liver vitamin A stores were not significantly affected by fat deficiency. As in the previous experiment (Morton & Horner, 1961) it was not possible to differentiate between impaired absorption and storage of vitamin A and its subsequent rate of utilization. Experiments to determine vitamin A utilization specifically have been done with fat-deficient animals, and will be reported elsewhere. In females, however, absence of cottonseed oil from a diet containing cholesterol caused a marked and significant decrease in liver vitamin A.

This sex difference differs from that described by Green *et al.* (1957), which was that the addition of cholesterol to a diet containing adequate fat caused a lowering of liver vitamin A in males, but not in females.

Differences emerged in the percentages of administered vitamin A found in the livers. Morton & Horner (1961) gave the animals 8800 i.u. of vitamin A and at the end of the experiment the livers of males contained 8% of the intake and those of females 11%. In the present work the animals were given 6400 i.u. and 25% of this was found in the livers of males, whereas in female controls the livers contained 16% of the intake as against 8% for deficient females.

These results might appear to be in conflict with an earlier finding (Horner & Morton, 1960) that vitamin A absorption or storage is depressed in male rats by dietary cholesterol. In that investigation the diets contained 10% of arachis oil. Control males given 10 000 i.u. before being placed for 42 days on a vitamin A-free diet had 40% of the dose in their livers. Another group of male controls, which received 3360 i.u. as daily doses of 60 i.u. for 56 days and were then killed, retained 37% of the dose in their livers. Thus vitamin A absorption or storage by rats given adequate dietary fat was more efficient than in any of our fat-deficient animals, or in their controls which received very small amounts of cottonseed oil.

The body weights given in Table 1 support the work of Holman & Peifer (1960), showing that cholesterol accelerates the onset of fat deficiency, by causing earlier cessation of growth. The Table shows how the female rat is less responsive than the male to these effects of dietary cholesterol. Morton & Horner (1961) found that without dietary cholesterol females ceased to grow after 170 days at a mean weight of 133 g.; males after 160 days at 167 g. In the present work females grew for 110 days, to a mean weight of 124 g. and in fact attained a higher final body weight than males, which ceased to grow after 72 days and averaged 107 g. in weight.

In the experiments of Bromer (1953) cholesterol had no significant effect upon the growth of male or female rats fed diets free from fat or containing hydrogenated arachis oil (2·9%) for 14 weeks. These findings are at variance with the present work and that of Holman & Peifer (1960). Bromer did, however, find that dermal symptoms were aggravated by cholesterol in the sense that they appeared appreciably sooner in males than in females and were more severe.

Liver lipids. Exogenous cholesterol which was deposited in the livers in these experiments was all esterified, as was the extra endogenous cholesterol which accumulated in fat-deficient animals (Morton & Horner, 1961). Increases in the concentrations of cholesteryl esters in the livers of fat-deficient animals given cholesterol were much more marked in males than in females. Bromer (1953) made a similar observation. The cottonseed oil which we gave to control animals did not alter this effect in males, but reduced it in females. Okey & Lyman (1957), under very different experimental conditions, observed that cholesterol feeding caused a greater accumulation of total lipid and cholesterol in the livers of males than in females, irrespective of the nature of the dietary fat.

The accumulation of exogenous cholesterol in the livers was much greater than the increase of endogenous cholesterol caused by fat deficiency alone.

Liver-triglyceride concentrations were very similar in control and deficient males. In both cases the values were lower than in deficient males which received no cholesterol (group B). The triglyceride levels of control and deficient females were both very similar to the group D value. Therefore dietary cholesterol did not increase the liver-triglyceride levels of fat-deficient animals. However, when fed together with cottonseed oil it caused an increase in liver triglycerides about equal to that caused by fat deficiency.

Accumulation of cholesteryl esters was caused by dietary cholesterol, by lack of linoleate, or by a combination of these factors. In no case was there any tendency for cholesteryl dienoate and tetraenoate to maintain or increase their normal con-

centrations, indeed the concentrations of these esters invariably fell as total cholesteryl ester levels rose.

Similarly, triglycerides which accumulated in fat-deficient animals were predominantly esterified with monoenoic and saturated acids. However, in males dietary cholesterol caused an increase in the percentages of dienoic, trienoic and tetraenoic acids bound to glycerol, whether the diet contained fat or not (compare group A with group 1 and group B with group 2). In females these effects were less marked and triglyceride dienoate concentration was actually lower in group 4 than in D.

Dietary cholesterol decreased the concentrations of dienoic and tetraenoic acids, particularly the latter, in the phospholipids of control animals. Both these effects were much more marked in males in spite of the fact that they received the larger dosage of linoleate. Popják (1946) reported a similar effect on rabbit plasma, wherein feeding a low-fat diet containing cholesterol caused a decrease in the unsaturation of plasma phospholipids.

There is noticeable a relative constancy of liver-phospholipid concentrations. In this experiment and the earlier work of Morton & Horner (1961) the liver-phospholipid levels ranged from 9·5 to 14·5 mg./g. of liver, with six of the values between 12·0 and 14·5 mg./g. In the present work each group had a lower liver-phospholipid concentration than the corresponding group in the previous experiments (Morton & Horner, 1961), which did not receive cholesterol. This effect of cholesterol was most marked in control males (group A, 13·7 mg. of phospholipids/g. of liver; group 1, 9·5 mg./g.), and least evident in control females (group C, 14·5 mg./g.; group 3, 13·7 mg./g.).

Similarly, Patil & Magar (1960) observed that dietary cholesterol lowered liver-phospholipid levels in male rats fed with various fats at the 5% level for 10 months (e.g. animals fed with arachis oil: 37·5 mg. of phospholipids/g. of liver; arachis oil plus cholesterol: 30·4 mg./g.). The example cited was less dramatic than the effect of adding cholesterol to diets containing coconut oil, ghee or dalda but we wish to compare it with an experiment of our own. Horner & Morton (1960) fed young male rats with a diet containing arachis oil (10%) for 6 weeks; half the animals also received cholesterol (2%). The control and cholesterol-fed groups had liver-phospholipid levels of 15·1 and 14·6 mg./g. respectively (Horner, 1958). Cholesterol did not affect liver-phospholipid concentrations appreciably under these conditions, and the phospholipid levels were very similar to those of fat-deficient animals and their controls. Possibly liver-phospholipid levels are less responsive to dietary changes in young rats than in adults.

Trienoic:tetraenoic acid ratios. Dietary cholesterol accelerated the onset of essential fatty acid deficiency as judged by earlier cessation of growth, appearance of dermal symptoms and lower final body weight.

It is difficult to find a 'chemical' criterion of fat deficiency which shows that the syndrome is more severe in cholesterol-fed animals. Holman (1960) has used the trienoic:tetraenoic ratio as an index of the degree of fat deficiency. High values of this ratio are indicative of fat deficiency because the rise in trienoic acid characteristic of this condition is always accompanied by a fall in tetraenoic acid. Table 2 shows that the value of this ratio for total liver fatty acids is always higher in fat-deficient animals.

Comparison of each group in the previous experiment with corresponding groups in the present work indicates that dietary cholesterol consistently raised the ratio for total liver fatty acids. This second effect, which was smaller than that due to fat deficiency, was observed whether or not the diet contained cottonseed oil.

In every experimental group the highest trienoate:tetraenoate ratio was that of the triglycerides. Deficiency of fat invariably raised the ratio above the control value in all three classes of liver lipid. Dietary cholesterol always raised the corresponding value in phospholipid fractions, but did not exert this effect consistently in cholesteryl esters or triglycerides.

Trienoate:tetraenoate ratios in phospholipid fractions always approximated to the corresponding values for total liver lipids. This fact allows group 4 to be included in a discussion of these values as an index of degree of fat deficiency.

By the standard of phospholipid trienoate:tetraenoate ratios, deficient females (group D) were more severely deficient than males (group B), the value for females being higher by a factor of 1·20. The addition of cholesterol to the diet increased the ratio by a factor of 1·22 for deficient males but had no significant effect on the value for females. Thus a direct comparison of the triene:tetraene values for the phospholipids of deficient rats fed with cholesterol shows no notable difference between the sexes.

The phospholipid trienoate:tetraenoate ratios of female control groups C and 3 were higher than those of the corresponding groups of male controls by factors of 1·72 and 1·93 respectively. These differences may be due to the smaller weight of cottonseed oil given to female controls. The chosen dosage levels were based on the data of Greenberg, Calbert, Savage & Deuel (1950).

Holman (1960) has suggested that linoleic acid should provide at least the equivalent of 1% of the total calorie value of the food eaten by male rats if

fat deficiency, as judged by the heart trienoate:tetraenoate ratio, is to be prevented. Our male controls received linoleic acid equivalent to 1·5% of total calories (assuming a daily intake of 15 g. of the basal diet). Female controls obtained the equivalent of 0·5% of their calorie intake as linoleic acid (food consumption 12 g./day).

Holman (1960) has also shown that a triene:tetraene ratio in plasma, erythrocytes or heart tissue less than approximately 0·4 indicates that the minimum requirement of linoleate has been met. This criterion cannot be applied to other tissues. Ratios based on the data of Rieckehoff, Holman & Burr (1949) [the data were recalculated and amplified with correction formulae derived from the spectral constants given by Holman (1957) for polyunsaturated acids isomerized by the method of Holman & Burr (1948)] indicate that in male rats given 100 mg. of corn oil per day, the value for heart (0·27) was considerably lower than that of liver (1·16) or any other tissue examined (i.e. kidney, brain, skin, liver, muscle and depot fat). In fat-deficient animals the value for heart was 4·30, higher than in any other tissue. The liver value in fat deficiency was 2·03. The increase in the heart trienoate:tetraenoate ratio was a much more sensitive indicator of fat deficiency than the corresponding change in the liver. (The second most responsive tissue 'indicator' was skin with ratios of 0·79 in controls and 3·86 in deficient animals.)

Similarly in an experiment carried out under very different conditions (Peifer, Janssen, Ahn, Cox & Lundberg, 1960) the trienoic: tetraenoic ratios given for heart lipids were consistently lower than the values which can be calculated for total liver lipids (e.g. heart, 0·33, liver, 1·49 in a group of hypercholesterolaemic male rats fed ethyl oleate).

Dienoic:tetraenoic ratios. Dietary cholesterol invariably raised the dienoic:tetraenoic ratios in total liver lipids and in the phospholipids and cholesteryl esters. The opposite effect was consistently observed in triglycerides. The changes did not depend on whether the diets contained cottonseed oil or not. They suggest that dietary cholesterol impairs the conversion of linoleate into arachidonate, an inference made by Klein (1959).

If group B is compared with group 2 and group D with group 4 the degree of this effect is seen to be virtually identical in deficient males and females; cholesterol increased the diene:tetraene ratio for phospholipid fatty acids by a factor of 1·4. There is a sex difference in the degree of this effect of cholesterol in control animals, but interpretation of its significance is complicated by the extra variable of linoleate dosage. Diene:tetraene ratios can be calculated from the data of Patil & Magar (1960). The addition of cholesterol to four diets each containing 5% of a fat consistently raised the diene:tetraene ratio in cholesteryl esters and lowered it in the triglycerides of livers of male rats. Our results obtained under very different experimental conditions confirm these findings. In the Indian experiment, however, cholesterol did not significantly change the diene:tetraene ratio in the phospholipids.

Females were more resistant to fat deficiency than males by several criteria. They continued longer to put on weight on a fat-free diet, and showed milder dermal symptoms. Dienoic and tetraenoic acids comprised a higher percentage of their total liver fatty acids (3·44% in group D; 2·59% in group B). Nevertheless by another criterion females were more deficient than males in that their liver triene:tetraene ratio was higher. Mead, Slaton & Decker (1956) have shown that in male mice linoleate oxidation is increased by fat deficiency, but the sexes were not compared.

Fat-deficient female rats were more resistant than males to the additional stress of dietary cholesterol. They grew for a longer period and became heavier than deficient males. Again dienoic and tetraenoic acids accounted for a greater percentage of the total liver fatty acids in females (> 2·61%) than in males (2·08%). By the tests of liver triene:tetraene and diene:tetraene ratios there was no sex difference in cholesterol-fed deficient rats. According to Lynn & Brown (1959) feeding cholesterol to rabbits, but not rats, stimulates the oxidation of all fatty acids by mitochondria, especially the unsaturated fatty acids. In rats cholesterol did not speed up the oxidation of linoleate by liver.

Feeding cholesterol caused a much greater deposition of cholesteryl esters in the livers of deficient males than of deficient females. The weight of dienoic and tetraenoic acids esterified to cholesterol in group 2 was 1·07 mg., equivalent to 27·4% of the total dienoic and tetraenoic acids in the liver. In group B the corresponding values were 0·27 mg. and 6·6%. If the essential fatty acids esterified to exogenously derived cholesterol were partially or wholly metabolically inert, i.e. unavailable to other classes of lipids or to other tissues, their deposition would accelerate fat deficiency. Further, their greater accumulation in males would explain why females are more resistant to a diet containing cholesterol and lacking linoleic acid.

Our experiments have revealed sex differences in the fat-deficiency syndrome and the effects of cholesterol, but do not lead to full explanations. The rates of turnover and the interconversions of the individual fatty acids in the various classes of lipids must be determined under differing dietary conditions. A beginning has been made by Dittmer & Hanahan (1959), who have studied the liver lipids

of normal male rats in this way, and Fulco & Mead (1959) have shown how eicosa-5:8:11-trienoic acid arises in fat-deficient rats.

SUMMARY

1. The liver vitamin A stores of fat-deficient male rats fed on a diet containing cholesterol (1%) did not differ significantly from those of controls which received linoleic acid.

2. The liver vitamin A stores of fat-deficient female rats fed on a 1% cholesterol diet were 48% lower than those of their controls which had been given linoleic acid.

3. The addition of cholesterol to a fat-free diet aggravated fat deficiency as judged by more rapid cessation of growth at a lower body weight. Both effects were appreciably more pronounced in males than in females.

4. Cholesterol-supplementation of the diet caused much larger accumulations of cholesteryl esters in livers than are caused by fat deficiency alone. The effect was much greater in males. Linoleic acid (100 mg./day) did not alter the severity of this phenomenon in males; a smaller dose (25 mg./day) reduced the effect considerably in females.

5. The rise in liver triglyceride concentration caused by fat deficiency was not altered by adding cholesterol to the diet. Cholesterol caused an increase in the concentrations of polyunsaturated acids in triglycerides, both in deficient animals and their controls. This effect was more pronounced in males.

6. Dietary cholesterol decreased the concentrations of dienoic and tetraenoic acids, particularly the latter, in the liver phospholipids of control animals. Both effects were appreciably greater in males than in females.

7. Dietary cholesterol consistently raised the triene:tetraene ratio of liver phospholipids.

8. Dietary cholesterol invariably raised the diene:tetraene ratio in total liver lipids, phospholipids and triglycerides, and lowered this value in the triglycerides. These effects occurred both in deficient animals and in controls.

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Ester-Linked Long-Chain Fatty Acids of Nervous Tissue

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It has long been recognized that nervous tissue contains a high proportion of lipid which on hydrolysis gives long-chain fatty acids in substantial amounts (Thudichum, 1901). Extracts of this lipid comprise a number of compounds that are now well characterized; the quantities in which these are present, and their chemical structures, have in many cases been elucidated (see LeBaron & Folch, 1957). Although the results of early qualitative work and of later quantitative studies on the acids from isolated fractions of different lipids extracted from nervous tissue have appeared in the literature, no survey of the nature and distribution of these acids in total lipid extracts of nervous tissues had been reported before the account by Johnston & Kummerow (1960) of the acids in two

samples of whole-brain lipid. Much of the acid is combined in ester linkage, as in the glyceryl-phosphatidyl compounds, the balance occurring largely in the form of amides, as in sphingomyelin. Small amounts of apparently free acid (Laser, 1949) were isolated from brain and characterized as octadec-11-enoic acid by Morton & Todd (1950).

Lecithins and kephalins from many sources contain C_{16} and C_{18} acyl residues, extending in the even-numbered series to C_{24} and even C_{26} (Klenk & Montag, 1958*a*, *b*). Acyl groups in the plasmalogens of ox-heart lipid are of similar chain length (Klenk & Krickau, 1957). Lipids nearly always contain unsaturated acids, often with more than one double bond in the molecule. Oleic acid and arachidonic acid were the only unsaturated acids that Levene & Rolf (1922*a*, *b*) could isolate from lecithin and kephalin of brain; they were unable to detect the corresponding dienoic acids. Arachidonic acid is an important component of lecithins from tissues other than the nervous system; with suprarenal gland or liver as the source, it accounts for over a fifth of the acids obtained by hydrolysis of the lecithin (Ault & Brown, 1940). Klenk & Böhm (1951) showed that hydrolysis of brain phospholipids yielded also C_{20} and C_{22} acids with up to four double bonds in the shorter-chain and six in the longer-chain acids. Further work in Klenk's laboratory clarified the structure of still more of the acids from brain phospholipid (Klenk & Bongard, 1952; Klenk & Lindlar, 1955*a*, *b*).

Fatty acids and their α-hydroxy derivatives occur as amides in the phospholipid fraction of nervous tissue in sphingomyelin, and in the cerebrosides, sulpholipids and ganglioside fractions of brain (Deuel, 1951). Acids liberated by hydrolysis of these are generally of higher molecular weight than those from the esters, and chains of 24 and 26 carbon atoms were found in the acids from cerebrosides (Klenk, 1928).

Knowledge of the acids incorporated into lipids of the nervous system has been limited in the past by the difficulties of their quantitative separation. Although countercurrent partition and paper chromatography gave new impetus, it was not until the methods of vapour-phase chromatography had been developed that semi-microanalytical work could be undertaken without recourse to laborious and imperfect quantitative techniques. In the work described below, a preliminary survey of the nature and quantities of ester-linked acids in some different regions of the human nervous system was undertaken, without attempting to relate the acids to chemically distinct lipid fractions. Striking differences between the acids in sciatic nerve and those in spinal cord and brain have been found. Within the brain, white and grey regions also showed points of contrast.

MATERIALS AND METHODS

Normal unfixed human brain and other nervous tissue from adults of either sex and from 38 to 81 years old was obtained at post-mortem examinations. Fatty acids for the preparation of methyl esters as marker materials were of laboratory-reagent grade (British Drug Houses Ltd. or L. Light and Co. Ltd.). Solvents and other substances were of A.R. quality wherever possible.

Extraction of lipids from brain and spinal cord was carried out by the method of Folch, Lees & Sloane-Stanley (1957); the weighed sample, chopped with scissors, was homogenized in 19 ml. of chloroform–methanol 2:1 (v/v) mixture/g. and the supension filtered. The filtrate was washed with 0·2 vol. of water, the phases were separated by centrifuging and the upper (aqueous) layer was rejected. Solvent was removed from the remainder in a rotary evaporator at 50° under reduced pressure, and residual lipid was dried to constant weight over $CaCl_2$ and paraffin wax in an evacuated desiccator. Sciatic nerve was freeze-dried and extracted with chloroform–methanol mixture, followed by washing etc. as above.

The fatty esters were hydrolysed by homogenizing the lipid (about 100 mg.) in 22·5 ml. of ethanol, mixing with 2·5 ml. of aq. 10N-KOH and agitating for 30 min. in a water bath at 38° by passing a stream of nitrogen through the suspension.

To separate the fatty acids formed, the hydrolysates were diluted with 30 ml. of water and acidified (Congo-red paper) with approx. 20 ml. of 2N-HCl; the organic acids were extracted by shaking gently first with ½ vol. and then with ¼ vol. of light petroleum (b.p. 40–60°). The combined light-petroleum solutions were washed with 0·5 vol. of 0·1N-HCl, separated, and extracted with 0·5 vol. of aq. 0·05N-NaOH by gentle inversion. The light-petroleum layer was stratified centrifugally and rejected, and the aqueous solution acidified (Congo-red paper) with an equal volume of 0·1N-HCl and twice extracted as before with light petroleum. The extracts were combined, washed with 0·5 vol. of 0·1N-HCl, separated, dried over Na_2SO_4, filtered, and evaporated under nitrogen; the acids remaining were dried *in vacuo* over $CaCl_2$ and paraffin wax to constant weight.

The mixtures of acids so obtained were converted into methyl esters by interaction in diethyl ether with diazomethane; this was prepared (De Boer, 1954) in a mixture of ether (2 ml.) and ethanol (3 ml.) in Quickfit B10 apparatus from 200 mg. of *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide (Diazald, Aldrich Chemical Co.) by reaction with 1 ml. of aq. 40% (w/v) KOH. The diazomethane was distilled with the ether (James & Webb, 1957*a*) into a solution of about 100 mg. of fatty acid in 1·5 ml. of ether. A trap consisting of a drying tube loosely packed with paper tissue damped with crude oleic acid absorbed escaping excess of diazomethane. Centrifuge tubes containing the reaction mixtures were placed in 150 ml. of ice-cold water in a fume chamber, until they had reached room temperature, when the ether was evaporated under nitrogen. Methyl esters required as reference substances in gas chromatography were prepared on a larger scale by leaving overnight at room temperature a solution of the acid in 25 vol. of methanol containing 2% (v/v) of H_2SO_4. Most of the methanol was distilled off, the residue was diluted with 5 vol. of water, and the ester was extracted with light petroleum. The extract was dried over Na_2SO_4, the solvent was

evaporated off and the esters were distilled under reduced pressure (approx. 0·4 mm. Hg) from an oil bath. Final purification was achieved by gas chromatography on a preparative scale with a column 2·5 cm. in diameter and 50 cm. long, packed with Apiezon L-coated Celite. With this column a full-flow catharometer detector designed for the purpose with directly heated Thermistor beads (Stantel Type P15, Standard Telephones and Cables Ltd., Footscray, Kent) was used. Currents in detector and reference Thermistors (the latter in a stream of pure carrier gas at the temperature of the column) were controlled at the same value by transistor stabilizers, and a balanced transistor amplifier was made to compare the potentials across the Thermistor elements. The carrier gas was nitrogen. Samples were collected by passing the effluent stream through drying tubes loosely packed with cotton wool washed with diethyl ether and moistened with methanol. Tubes were changed as indicated by the detecting system to collect predetermined peaks. The cotton wool was then extracted with diethyl ether, which was finally evaporated to leave pure ester. Methyl esters of lauric, myristic, palmitic, stearic, oleic, linoleic, arachidonic and clupanodonic acids were prepared. In the treatment of unsaturated acids, temperatures were not allowed to exceed 50° except under nitrogen or *in vacuo*.

Analytical methods. A colorimetric method (Stern & Shapiro, 1953) was used to estimate acyl-ester groups in the hydrolysis experiments described below, with tristearin as the standard.

Vapour-phase chromatography (see James, 1960; Rose 1959; Farquhar, Insull, Rosen, Stoffel & Ahrens, 1959) in packed columns 130 cm. high and of bore 3–4 mm. was applied for analysis of 0·02–1·0 μl. samples of mixtures of methyl esters. The apparatus was essentially as described by James, Lovelock & Piper (1959), with argon as the carrier gas and ^{90}Sr as the radioactive source in an ionization detector (Lovelock, 1958) in which a modified sparking plug was used as collector electrode. Lovelock's amplifier and high-tension supply unit were used in conjunction with a recording milliammeter. Chromatograms were also recorded with a simple battery-operated circuit in which current was limited by a resistance of $5 \times 10^9\ \Omega$; signals were derived from a load resistor of $10^7\ \Omega$ in series with the detector cell, battery and limiter, and were fed directly to a high-impedance recording millivoltmeter (Kent, Multelec Mark 3).

Commercial Celite 545 was sieved into fractions passing 85–100 or 100–120 mesh screens, calcined at 300° for 3 hr., washed with HCl, water and 1% KOH in methanol for use as support for the stationary phase. This was Apiezon L hydrocarbon grease, Reoplex 400 (Stoffel, Insull & Ahrens, 1958), or a polyester prepared in the laboratory, e.g. polydiglycol succinate and adipate (Lipski, Landowne & Godet, 1959). Working temperatures were in the range 165–205°, with gas-flow rates of 25–55 ml./min.

The identities of eluted esters were provisionally established from retention-volume data obtained with different stationary phases, with pure methyl palmitate or stearate as reference substance. Confirmation was obtained by direct comparison with the methyl esters prepared as above. Relative quantities in the mixtures of esters examined were assessed by planimetry of the chromatograms. Linearity of response within the limits of load and polarizing potential used was within 5% when tested with mixtures of palmitic and stearic esters of known compositions.

RESULTS

The yields of total lipid extracted from whole brain and from the seven regions of the nervous system are shown in Table 1. Considerable variation in content of fat from one region to another is evident. In the same Table are recorded the quantities of fatty acid obtained after hydrolysis of the esters.

The efficiency of the procedure chosen for hydrolysis (see Dawson, 1954) was investigated and confirmed in experiments in which the decrease in ester equivalent of the biologically derived and of other fatty esters was followed during the course of reaction, with results indicated in Table 2. The acyl-ester content of the reaction mixture was estimated by applying the hydroxamic acid method to acidified samples. It was necessary to estimate the ester as rapidly as possible after acidifying, since in the acid solution re-esterification occurred at a steady rate of 2·6%/hr. at room temperature. At 0°, no measurable change occurred in 2 hr. Notable lability of the acyl groups and uniformity of the speeds of hydrolysis were revealed. The standard deviation in replicate determinations of

Table 1. *Yields of total lipid extracted from nervous tissues and of fatty acids obtained by mild alkaline hydrolysis of the lipid*

S.D. = Standard deviation (no. of samples in parentheses).

		Brain							
				Grey					
		Whole	White (corpus callosum)	Cortex	Thalamus	Caudate nucleus	Putamen	Spinal cord (thoracic)	Sciatic nerve
Lipid content (g./100 g. of fresh tissue)	Mean value	10·2	21·4	8·3	16·6	12·3	9·6	19·9	24·5
	S.D. (6)	1·5	3·4	1·9	1·1	4·0	2·8	6·4	8·1
Fatty acid content (g./100 g. of fresh tissue)	Mean value	2·32	2·04	1·64	2·85	2·46	1·74	3·59	8·07
	S.D. (6)	0·27	0·62	0·56	0·59	0·65	0·23	2·24	2·76

acyl ester was 4% with tristearin and 6% with a typical sample of hydrolysis mixture. Slightly higher extinctions (e.g. 0·209, compared with 0·207) were consistently given by tristearin added to hydrolysis mixtures, and observations were corrected in this ratio.

To check the method described above for extracting the acids formed by hydrolysis, diluted and acidified hydrolysis reaction mixtures were shaken serially with ⅓ vol. quantities of light petroleum, and each extract was washed with 0·1 N-HCl and evaporated; the weights of the fatty acid residues were determined. Of the total acid obtained from three serial extractions, 92·8% (w/w) was present in the first, 5·6% in the second and 1·6% in the third extract.

A classification of the fatty acids obtained from nervous tissues and the relative quantities in which they were present are summarized in Table 3. Lower acids (C_8–C_{12}) were found only in traces in all the regions of brain studied, but were quite prominent in spinal cord and still more so in sciatic nerve. Myristic acid occurred in all the samples, the quantities in corpus callosum and in cord being 1·6% of total acid; sciatic nerve contained over 4%. An insignificantly small proportion of these lower acids was unsaturated. From 15 to 30% of the acids were of the C_{16} straight-chain type in all the lipids analysed, palmitic acid preponderating. Palmitoleic acid accounted for an eighth or less of this group except in the nerve (where it constituted nearly a third) and in spinal cord.

The C_{18} acids comprised approximately one- to two-thirds of the total in all cases, but stearic acid itself was unevenly distributed, its amounts ranging from 5% in sciatic nerve to 23% in whole brain. The largest class seen in every region was that of the octadecenoic acids; these were not resolved into position isomers and are referred to simply as oleic

Table 2. *Time-courses of hydrolysis of lipids*

The lipid (0·5–1·2 g.) was treated with 25 ml. of ethanolic 90% (v/v) N-KOH at 38°. The results are expressed as percentages of acyl ester unhydrolysed (value at zero time = 100%).

Time (min.)	2·5	5	10	15	20	25	30
Whole-brain lipid	48	27	12	6·0	5·0	4·0	4·0
White-matter lipid	42	17	5·0	2·0	4·0	3·0	3·0
Grey-matter lipid	40	20	6·0	4·0	3·0	3·0	3·0
Spinal-cord lipid	35	17	6·0	5·0	4·0	3·0	3·0
Sciatic-nerve lipid	57	25	10	7·0	5·0	4·0	4·0
Human-liver lipid	37	15	9·0	8·0	7·0	6·0	6·0
Triolein	64	24	12	8·0	6·0	—	—
Tristearin	42	13	3·0	1·0	1·0	—	—
Cholesterol oleate	42	23	19	2·0	2·0	—	—

Table 3. *Mean values, as percentage of total acid, for ester-bound acids in total lipid from nervous tissue*

+, < 0·05; + +, < 0·50, > 0·05.

Fatty acid	No. of carbon atoms	No. of double bonds	Brain						Spinal cord (thoracic)	Peripheral nerve (sciatic)
			Whole	Corpus callosum	Cortex	Caudate nucleus	Thalamus	Putamen		
Caprylic	8	0	+	+	+	+	+	+	+	+
Capric	10	0	+	+	+	+	+	+	0·1	1·7
Lauric	12	0	+	0·1	+	+	+	+	0·2	1·0
Myristic	14	0	0·1	1·6	0·7	+	0·2	+	1·6	4·2
Palmitic	16	0	24·0	15·0	28·7	22·5	20·5	25·3	14·3	19·2
Palmitoleic	16	1	3·2	+ +	+ +	+ +	+ +	+ +	3·9	8·3
	16	2	+ +	+ +	+ +	+ +	+ +	+ +	0·6	1·5
Stearic	18	0	23·0	16·2	15·5	21·0	19·1	20·7	7·7	5·0
Oleic	18	1	32·6	39·4	22·0	25·1	33·9	30·4	51·6	49·7
Linoleic, linolenic	18	2, 3	+ +	3·5	3·1	5·0	3·7	0·1	1·1	8·6
	20	1–3	3·9	10·7	5·9 }	13·7	{ 6·7	5·8	6·6	0·7
	20	4–6	6·5	9·2	6·0 }		{ 8·5	9·6	7·4	0·2
	22	1–4	3·6	1·1	6·4 }	8·9	{ 1·4	0·7	4·0	0·2
	22	5, 6	2·0	3·2	11·8 }		{ 3·1	4·3	1·0	0·2
	24	1–5	+ +	+ +	+ +	3·9	3·3	2·0	0·2	0
Total			98·9	100·0	100·1	100·1	100·4	98·9	100·3	100·5

acid. It formed about a third of the brain acids and half of those from the cord and nerve. The C_{18} group included *n*-octadecadienoic and traces of the trienoic acids.

With increasing chain length, unsaturation increased. Only traces of arachidic acid and behenic acid were detected, the latter only in a few brain samples; yet the C_{20} class accounted for 10–20 %, the C_{22} class for 4–18 % and the C_{24} class for up to 4 % of the esterified fatty acids of brain and cord lipids. Arachidonic acid was the chief representative of the C_{20} group, constituting up to 8 % of the total acid. Among the C_{22} acids, members with up to three double bonds were scarce, but the more unsaturated acids formed about 6 % of the whole, with clupanodonic and docosahexenoic acids as the major representative. The C_{24} acids were lower in proportion and polyenoid in character.

Although spinal cord and brain showed substantial and similar quantities of the C_{20} acids, peripheral nerve yielded less than 1 %. The C_{22} acids formed 18 % of the yield from cortex, 9 % of that from the caudate nucleus, but only 5 % in other samples, except sciatic nerve, in which only traces were seen. Although C_{24} acids formed 4 % of the acid in the caudate nucleus and thalamus, they occurred to a less extent in other regions of the brain, were barely detectable in spinal cord, and were not seen at all among the acids derived from sciatic nerve. C_{15}, C_{17} and C_{19} acids were found as minor components in most of the samples.

DISCUSSION

The yields of lipid found for whole brain and for corpus callosum are close to the values reported by Folch & LeBaron (1957) for whole brain and white matter, whereas cortex showed a higher content than that quoted by these authors. Their range for the lipid content of peripheral nerve (16·7–21·5 g./100 g.) is also lower than the mean (24·5 g./100 g.) recorded above for human sciatic nerve, which is difficult to separate from connective tissue, as discussed below.

The comparatively mild conditions used for hydrolysis, for extraction of acids and for the formation of their methyl esters were shown to be quantitative, and do not expose the labile polyenoid acids to serious risk of change. Neither was such change found when substances of similar character were submitted to gas chromatography under the conditions obtaining in the present work (James & Webb, 1957*b*; Stoffel *et al.* 1958). It is considered, therefore, that the acids found are true constituents of the original tissue. The small quantities of residual and apparently stable acyl ester appearing after 30 min. hydrolysis (see Table 2) may be, at least in part, artifacts, for the quantities involved are comparable with the error of the method.

Although essentially quantitative recovery of the acids formed by hydrolysis was demonstrated, the product was still accompanied by impurities, e.g. cholesterol. Esters made by methylation of the acids from brain and spinal cord, but not from sciatic nerve, were yellow solids with melting points sometimes as high as 60°. After distillation, the ester mixtures were almost colourless and melted below 30°. The weights of acid recorded in Table 1 include the weights of contaminants, and are about 16 % in excess of the theoretical values calculated from the ester equivalents of the lipids and the mean molecular weights for the acid mixtures, assessed from their compositions. Nevertheless, crude and distilled esters gave identical gas chromatograms when somewhat larger samples of the crude ester were taken. The contaminants therefore did not emerge from the columns; this is consistent with the view that the impurities are polar substances of high molecular weight.

Evaluation of the purity of the acids extracted is difficult. No reliance can be put on titrimetric or gravimetric methods, since the absence of acidic groups from the impurities cannot be assumed. Similarly determination of ester equivalent after methylation is not admissible. Molecular distillation could be used only with caution, because highly unsaturated acids easily form resins when heated. Comparison of peak areas on chromatograms with those given by pure substances is not accurate because of the difficulty of applying samples of the necessary size reproducibly to the column.

Johnston & Kummerow (1960) treated whole-brain lipid by prolonged acid methanolysis, and obtained values for the fatty acids combined both in amide and ester linkage. There is general agreement between their values and those shown above for whole brain, but comparison in detail is precluded by the inclusion in their values of the amide acids, known to be of longer average chain-length.

The results in Table 3 reveal a sharp difference in several respects of peripheral nerve from the other nervous tissue. Peripheral nerve contains appreciable amounts of myristic and of lower acids, which occur to a minor extent at the other sites. Palmitoleic acid is a prominent component in the nerve, but is relatively insignificant in brain. Equally sharp in contradistinction from brain is the low quantity of stearic acid in sciatic nerve, offset by a greater abundance of oleic acid and of linoleic acid. The paucity of higher acids is another notable characteristic of the nerve lipid. The results for sciatic nerve resemble those for adipose tissue (Table 4), which also is clearly different in its acid composition from the lipids of brain and spinal

Table 4. *Percentage composition of esterified fatty acids from human adipose tissue and sciatic nerve*

Fatty acid	Adipose tissue (Cramer & Brown, 1943)	Adipose tissue (Moore & Cook, 1959)	Sciatic nerve
Lauric	1	1–2	2·5
Myristic	2–6	3–5	3–7
Myristoleic	1	—	0·5
Palmitic	25	22–31	13–26
Palmitoleic	5–8	5–14	4–11
Stearic	5–9	2–15	2–9
Oleic	45–47	42–51	43–55
Linoleic	10–11	1–6	8–10
Total of means	99·5	100	96

cord. This supports the hypothesis that much of the lipid extracted from the peripheral nerve derives from associated connective tissue, and is not properly of nervous origin.

Spinal cord approximates to the nerve in containing lower acids, although in far smaller quantities. Its poverty in palmitic acid gives it a place with corpus callosum, but in its contents of palmitoleic acid, stearic acid and the highly unsaturated C_{22} acids it is intermediate between the nerve and brain. The C_{20} acids in spinal cord do not differ significantly from those in brain.

The cerebral cortex differs from corpus callosum in containing nearly twice as much palmitic acid but only half as much oleic acid. There is also a differing distribution of the longer-chain acids, cortex showing a smaller total of C_{20} but a relative excess of C_{22} acids.

The three nuclear areas taken seem not to differ markedly among themselves in acid contents; they resemble the grey rather than the white portion of brain, reflecting the anatomical relationships. A common finding for the brain fractions was the low quantity of palmitoleic acid and the proportion, approximately 25 %, of C_{20}–C_{24} unsaturated acids.

The high proportion of sphingomyelin in nerve lipid (Carter, Haines, Ledyard & Norris, 1947; Johnson, McNabb & Rossiter, 1948), and the predominance of higher (C_{20}–C_{24}) acids in this compound (Thannhauser & Boncoddo, 1948) give reason for believing that only ester-bound acids were assayed in the present work, since these acids were barely detected in lipid from sciatic nerve. Such higher acids found in the brain and spinal cord thus appear to be truly of ester origin. Further, fatty acid amide linkages are known to be stable to alkali at 37°, and the conditions described for the hydrolysis of amide linkages are very different from those used here for the fission of ester groups. Prolonged heating in strongly acidic methanol was employed by Klenk (1927) and by Thannhauser & Boncoddo (1948). It is therefore probable that no significant splitting of amides occurs in the gentle hydrolysis of the esters described above.

SUMMARY

1. The esterified fatty acids in lipids extracted from normal human whole brain, from five regions of brain and from spinal cord and sciatic nerve were liberated by hydrolysis and examined as methyl esters by gas chromatography. Yields of lipid and of fatty acid were measured, and the composition of the fatty acid fraction was determined.

2. The fatty acid pattern of sciatic nerve was distinct from that of the other samples, and resembled that of body fat.

3. The compositions of fatty acid mixtures from brain varied little from site to site. The approximate values were palmitic acid 20 %, stearic acid 20 %, C_{20} acids 16 %, C_{22} acids 5 % and C_{24} acids 4 %, the remaining 35 % being mostly oleic acid. Lower acids (up to C_{14}) were barely detectable.

4. Fatty acid values for spinal-cord lipid lay between those of brain and of the nerve.

For most helpful discussions on the gas-chromatographic method the author is greatly indebted to Dr A. T. James, Dr J. E. Lovelock and Miss J. Webb. For the suggestion that this method should be applied to problems in neurochemistry the author thanks Professor R. H. S. Thompson. The Multiple Sclerosis Society gave a grant with which apparatus used in this work was constructed in the laboratory. Miss Pamela Fowler assisted throughout in the chemical and analytical work.

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The Composition of Collagen from Subcellular Fractions of Guinea-Pig Granuloma Tissue

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Investigations of the amino acid composition of vertebrate collagens have been largely confined to preparations directly derived from whole tissues of high collagen content (Eastoe & Leach, 1958). Most studies have been made on the insoluble fibrous protein remaining after treatment of the tissue with solvents which could remove other tissue components with minimum degradation of the collagen (Bowes & Kenten, 1948). Such preparations would be expected to consist largely of extracellular collagen fibres with characteristic 640 Å striations. Recently analyses have been made of collagen fractions extracted from tissues by neutral sodium chloride and slightly acid sodium citrate solutions (Bowes, Elliott & Moss, 1955; Jackson, Leach & Jacobs, 1958). These soluble collagens represent only a small percentage of the total collagen, and much evidence, based on the incorporation of radioactive amino acids, has accumulated which shows that the neutral-salt-soluble fraction is synthesized before the insoluble fibres, which are probably formed by aggregation of the smaller particles in the soluble fraction (Harkness, Marko, Muir & Neuberger, 1954; Jackson, 1957; Green & Lowther, 1959).

The object of the work described here was to determine the composition of the protein material extracted by sodium chloride solution from three subcellular fractions of rapidly developing mesenchymal tissue from guinea-pig granuloma. It forms part of a more extensive investigation involving radioactive tracer studies of proline hydroxylation in the subcellular fractions and their examination by electron microscopy (Lowther, Green & Chapman, 1961; Chapman, 1961). Since the protein fractions had already been shown to contain hydroxyproline and to have solubility properties similar to neutral-salt-soluble collagen extracted from whole tissues, confirmation of the characteristic overall amino acid pattern would be important evidence of the intracellular synthesis of the collagen molecule. The very small quantities of protein, obtainable from a large bulk of granuloma tissue, required the adoption of a modified semi-micro method of amino acid analysis, based on resin chromatography, which is described in an Appendix to this paper.

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MATERIAL AND METHODS

Subcellular collagen fractions. The collagen fractions were isolated and purified by Dr N. M. Green and Dr D. A. Lowther (see also Lowther *et al.* 1961) by the following methods. Granulomata, produced by subcutaneous injection of carrageenin solution into guinea pigs, were removed 7 days later, immediately after killing the animals. The tissue was chopped finely with two 6 in. razor blades and serum proteins were largely removed by centrifuging a

suspension in 2·5 vol. of 0·25 M-sucrose at 1000*g* for 30 sec. The sediment was homogenized in more sucrose soln. at 0° in a glass tube fitted with a Teflon pestle driven at 1400 rev./min. via a flexible shaft.

Fractions of different particle size were separated by differential centrifuging (Schneider & Hogeboom, 1950) with the following sedimentation conditions: (*a*) nuclear fraction, 10 min. at 1000*g*; (*b*) mitochondria, 10 min. at 10 000*g*; (*c*) microsomes, 35 min. at 105 000*g*. For each fraction the sediment was resuspended in sucrose soln. and again centrifuged. The washings were added to the bulk of the supernatant fluid.

Collagen in the three sedimented fractions from differential centrifuging was extracted by suspending them overnight at 4° in 0·2 M-NaCl–6·7 mM-ethylenediaminetetraacetic acid (sodium salt), pH 7·4. Each suspension was centrifuged at 105 000*g* for 30 min., to remove insoluble matter, and the NaCl concn. of the supernatant solution was raised to 20% (w/v) to precipitate the collagen. The precipitate was centrifuged and the extraction and precipitation were repeated. Finally the precipitate was dissolved in 0·05 M-acetic acid and dialysed overnight against 0·05 M-acetic acid.

Analytical methods

Total N. This was determined by the micro-Kjeldahl method.

Hydrolysis. This was carried out under N_2 in sealed tubes with 6 N-HCl at 100° for 48 hr.

Protein determination. Approximate values for protein were obtained by the method of Lowry, Rosebrough, Farr & Randall (1951) with gelatin for calibration.

Amino acid analysis. This was carried out by using a semi-micro modification (Eastoe, 1960) of the method of Moore & Stein (1951) as described in the Appendix.

RESULTS

From 200 g. of granuloma, approximately 0·7 mg. of microsomal collagen was obtained, of which 0·55 mg. was available for amino acid analysis. After a preliminary determination of protein by the method of Lowry *et al.* (1951), the hydrolysate was divided between four hydroxyproline determinations and a single determination of the other amino acids. The larger quantities of the nuclear and mitochondrial fractions (Table 1) permitted, in addition, the determination of total nitrogen.

The values for the amino acid contents of the subcellular collagen fractions are given in Table 1. Since the total nitrogen content of the microsomal fraction was unknown, all results were calculated as numbers of amino acid residues per thousand total residues recovered. This basis for expressing analytical values facilitates comparison between protein samples with different proportions of non-protein impurities and is valid provided that recovery of amino acids is substantially complete (Eastoe & Leach, 1958). The recoveries of total nitrogen, 104·6 and 103·4% for nuclear and mitochondrial collagen respectively, were high. This tendency has been noticed previously and its possible causes were discussed (Eastoe, 1955). The present recoveries are greater than those found previously but suggest complete recovery of total nitrogen within the limits of cumulative experimental error to be expected from the total nitrogen and amino acid determinations (Eastoe, 1960).

The appearance of the elution curves for all the collagen fractions was similar to that for microsomal collagen (see Appendix, Figs. 2 and 3). Good separations were obtained for all peaks except the pairs hydroxyproline–aspartic acid and tyrosine–phenylalanine. For this reason, hydroxyproline was determined separately. The presence of its yellow peak of low extinction was considered unlikely to cause substantial errors for aspartic acid. Unfortunately the hydroxylysine and histidine peaks from the column (17 cm. × 0·4 cm.) were not resolved when the mitochondrial hydrolysates was examined and none remained for further work. With the microsomal fraction, this difficulty was overcome by modifying the technique (see Appendix). Small amounts of ninhydrin colour were noticed near the hold-up volume for the 100 cm. column and may have been attributable to cysteic acid corresponding to traces of cystine in the protein. Tryptophan, which is considered to be absent from collagen (Bowes & Kenten, 1947), was not determined.

The quantities of ammonia, recovered from all three hydrolysates of subcellular fractions, greatly exceeded the amide nitrogen content of collagens from whole tissue. This was probably due to the presence of ammonium salts as impurities in the reagents used for preparation of the fractions.

DISCUSSION

The neutral-salt-soluble portions of the nuclear, mitochondrial and microsomal fractions, obtained by differential centrifuging, showed all the typical features of the amino acid pattern of vertebrate collagens. Thus all three contained as much or almost as much hydroxyproline as did the collagen and gelatin samples previously analysed (Table 1). Hydroxylysine was also present in the nuclear and microsomal material, and, although it was not satisfactorily demonstrated in the mitochondrial fraction, the size of the combined hydroxylysine–histidine peak indicated that both could be present in the same proportions as in insoluble collagen. Failure to resolve this pair, when using the same technique as for the mitochondrial fraction, has been subsequently confirmed for a gelatin hydrolysate, known to contain both amino acids. All the subcellular fractions contained large amounts of glycine, alanine and proline and small amounts of amino acids with hydrocarbon or aromatic side chains, both features typical of collagen.

Table 1. *Composition of neutral-salt-soluble collagens from subcellular fractions of guinea-pig granuloma and from whole rabbit skin, insoluble collagen from ox bone, and gelatin from ox skin*

Values are given as numbers of amino acid residues per 1000 total residues. For abbreviations of amino acids see *Biochem. J.* (1957), **66**, 6.

	Neutral-salt-soluble collagen				Insoluble collagen from ox bone	Gelatin from ox skin
	Guinea-pig granuloma (present study)			Rabbit skin (Jackson, Leach & Jacobs, 1958)	(Eastoe, 1955)	
	Nuclear	Mitochondrial	Microsomal			
Ala	100·7	101·8	93·4	100·8	109·7	112·0
Gly	324	300	263	296	314	333
Val	20·5	24·1	29·1	26·9	21·2	20·1
Leu	25·5	29·0	41·6	29·7	27·9	23·1
Ileu	11·8	13·4	22·4	17·0	12·3	12·0
Pro	115·6	109·8	101·5	111·5	118·8	129·0
Phe	12·4	14·8	18·5	15·1	16·3	12·3
Tyr	3·2	4·8	11·3	5·6	2·9	1·5
Ser*	44·1	48·7	51·8	44·5	37·8	36·5
Thr*	20·2	24·8	31·5	27·4	19·7	16·9
Cy_2S_2	Trace	Trace	Trace	Trace	Trace	Trace
Met†	8·3	6·9	6·0	6·5	5·1	5·5
Arg	51·6	55·8	59·0	47·6	49·0	46·2
His	6·2	—	9·9	7·2	5·8	4·5
Lys	25·8	28·3	36·1	31·9	26·2	27·8
Asp	48·3	54·0	61·6	54·8	49·8	46·0
Glu	68·2	71·7	76·0	75·7	75·8	70·7
Hypro	104·4	100·0	79·6	97·1	100·8	97·6
Hylys	8·8	‡	7·6	4·8	6·4	5·5
Hydrolysate NH_3§	101	190	114	50·8	41·8	47·5
Kjeldahl N (mg.)	0·42	0·215	—	—	—	—
Total N recovered (mg.)‖	0·439	0·222	0·108	—	—	—
Recovery of N (%)	104·6	103·4	—	105·2	101·4	100·9
Total protein (Lowry *et al.* 1951) (mg.)	1·9	1·2	0·78	—	—	—
Total protein from N value (mg.)¶	2·27	1·08	0·55	—	—	—
Molar ratios Gly/Hypro	3·10	3·00	3·30	3·05	3·12	3·41
Molar ratios Ala/Hypro	0·97	1·02	1·17	1·04	1·09	1·15
Molar ratios Pro/Hypro	1·11	1·10	1·28	1·15	1·18	1·32

* Corrected for decomposition during hydrolysis.
† Sum of methionine and methionine sulphoxide peaks.
‡ Sum of histidine and hydroxylysine 12·4 residues/10^3.
§ Corrected for NH_3 formed by decomposition of serine and threonine.
‖ Sum of amino acid and ammonia N.
¶ Calculated from N recovered as amino acids plus amide N (assumed value, 41·8/10^3) assuming a total N content of 18·5%.

Small variations in the content of individual amino acids of vertebrate collagens (Eastoe & Leach, 1958) render difficult the selection of a standard set of values for comparison with the present series. Table 1 includes values for insoluble bone collagen from which certain impurities had been removed by extractive processes (Eastoe, 1955), collagen extracted from rabbit skin by neutral-salt solution (Jackson *et al.* 1958) and an alkali-processed gelatin. It has been suggested (Eastoe, 1955) that, with certain reservations concerning the arginine and amide contents, such gelatins may closely represent pure collagen in composition, since soluble and insoluble impurities have been removed in the pretreatment and extraction stages of manufacture respectively.

Nuclear granuloma collagen closely resembles both insoluble collagen and gelatin in composition, many of its amino acid values lying between those of these two proteins (Table 1) and differences seldom exceeding the probable experimental errors (Eastoe, 1960). The mitochondrial fraction is similar in composition to the nuclear material but contains 4–7% less of the typical collagen amino acids glycine, hydroxyproline and proline and correspondingly more of the less abundant residues. The microsomal fraction shows these departures to a greater degree, there being 15–20% less glycine, proline, hydroxyproline and hydroxylysine. This is

consistent with the presence of 15–20% of protein impurities in the microsomal fraction since the molar ratios of glycine, alanine and proline to hydroxyproline are within the range of other proteins in Table 1, whereas the amounts of some amino acids present in small proportions in collagen, e.g. tyrosine, leucine and isoleucine are significantly increased in the microsomal fraction. The neutral-salt-soluble fraction from whole rabbit skin is closely similar in composition to the present mitochondrial fraction extracted by that solution. Lowther *et al.* (1961) have shown that the microsomal fraction accounted for 7% of the total nitrogen but only 0·3% of the hydroxyproline of the granuloma, which indicates that this fraction contained only a small proportion of collagen and considerable amounts of other proteins. The contamination of the material extracted from the microsomal fraction with non-collagen material is likely to be due to the small proportion of collagen present in it.

Electron microscopy of thin sections of the entire fractions separated by centrifuging before extraction with salt solution has shown that considerable damage occurs to both nuclei and mitochondria owing to the severe treatment necessary to homogenize the tissue (Lowther *et al.* 1961). Large numbers of typical striated collagen fibres were present in the nuclear fraction and very few in the mitochondrial fraction. Both these fractions also contained large amounts of fine non-striated fibrils. The microsomal fraction was more homogeneous than the heavier fractions and consisted mainly of rough- and smooth-surfaced vesicles but it was apparently entirely free from both types of fibril.

This suggests that the neutral-salt-soluble collagen extracted from the microsomes may not occur as fibrils. Lowther *et al.* (1961) have shown that 6·5, 33 and 80% of the hydroxyproline of the nuclear, mitochondrial and microsomal fractions respectively are soluble in the salt solution. Thus the protein analysed accounted for all of the microsomal collagen, except for a small amount not precipitated with 20% sodium chloride solution and which was possibly water-soluble (Green & Lowther, 1959). Lowther *et al.* (1961) were able to co-precipitate neutral-salt-soluble collagens from nuclear and microsomal fractions, which indicates that the collagen of the microsomes is similar to and possibly identical with the neutral-salt-soluble collagen of the whole granuloma. If the collagen of the microsomes is non-fibrillar it would seem likely that this applies to the whole of the neutral-salt-soluble collagen of the cell.

Several workers have emphasized the importance of the neutral-salt-soluble fraction as representing an early stage in the formation of insoluble collagen. The present investigation shows that the composition of this soluble collagen from three subcellular fractions is closely similar to that of insoluble collagen. Thus a protein having the characteristic composition of extracellular collagen fibres is found within the cell, being probably synthesized, like other proteins, by the microsomes.

SUMMARY

1. The amino acid composition of subcellular fractions of collagen, obtained by differential centrifuging of homogenized guinea-pig granuloma and purified by extraction with 0·2M-sodium chloride solution, has been determined by using a semi-micro modification of the ion-exchange chromatographic method of Moore & Stein.

2. The collagens from the nuclear and mitochondrial fractions were closely similar in composition to both insoluble collagen from ox bone and neutral-salt-soluble collagen from rabbit skin. These fractions had been shown by electron microscopy to be heterogeneous and to contain both striated and plain fibres. Collagen from the relatively homogeneous microsomal fraction had the same overall amino acid pattern as insoluble collagen but with quantitative differences, suggesting the presence of 15–20% of protein impurities.

3. It is suggested that the non-fibrillar collagen, synthesized in the microsomes and which is almost completely soluble in 0·2M-sodium chloride solution, is similar to and possibly identical with the fibrils of insoluble collagen as regards amino acid composition.

I am grateful to Dr N. M. Green and Dr D. A. Lowther of the Department of Chemical Pathology, St Mary's Hospital, Paddington, for preparing the purified collagen fractions and for the opportunity to collaborate in this investigation. I should also like to thank Sir Wilfred Fish and Professor A. Neuberger, F.R.S., for their kind interest.

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APPENDIX

A Semi-Micro Method for the Determination of Amino Acids by Ion-Exchange Chromatography

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The method of protein analysis, introduced by Moore & Stein (1951) and based on separation of amino acids on ion-exchange columns eluted with buffer solutions of accurately controlled pH, has become widely adopted. Collaborative tests have shown that, provided sufficient care is taken with all details of technique, the accuracy of $\pm 3\%$, originally claimed, can be attained for most amino acids (Bender, Palgrave & Doell, 1959). In some fields of investigation, e.g. purified enzymes and microbial toxins, enamel matrix (Eastoe, 1960*a*) and subcellular protein fractions, it is difficult or tedious to obtain even the 2–4 mg. of protein required for analysis by the standard method. A semi-micro method suitable for 0·3–0·6 mg. samples of protein was developed and has been described in a preliminary communication (Eastoe, 1960*b*). It is based on the original method of Moore & Stein (1951) with the minor changes suggested by Eastoe (1955). The modifications necessary for the semi-micro method, together with an indication of the accuracy and resolution obtainable, are described briefly below. It is not considered that this modification represents the ultimate in small-scale protein analysis, but is an exploratory step in that direction.

MATERIALS AND APPARATUS

Resin, buffers etc. The same batch of Dowex 50 resin (200–400 mesh; nominal 12% cross-linking) was used as previously (Eastoe, 1955). Citrate and phosphate buffers, thymol, BRIJ 35, thiodiglycol, ninhydrin and ethylene glycol monomethyl ether were prepared or purified as before. Industrial spirit (74 over proof) has been found to be a satisfactory substitute for absolute ethanol in the preparation of the diluent solution. The semi-micro method is considerably more economical in ninhydrin and buffer solutions than the original method and it is therefore unnecessary to store large volumes of solutions.

Columns. The borosilicate glass tubes for the 15 and 100 cm. resin columns were 28 and 112 cm. in overall length respectively. They were selected for straightness and for an internal diameter of $4{\cdot}0 \pm 0{\cdot}1$ mm. (external diam. approx. 6·8 mm.). Each tube had a fused-in sintered glass disk (porosity 1 or 2), 2·0 cm. from its lower end, which was cut off at 45° and its tip fused to a small blob of glass. Care was necessary to ensure that the disk was fused to the wall all round but not fused solid itself. The portion of tube below the sinter did not normally remain filled with solutions which contained detergent. The tubes were fitted by means of rubber bungs into side-armed glass jackets of overall length 18 and 102 cm. respectively. The columns were filled with resin as described by Moore & Stein (1951) with additional precautions to prevent trapping of air bubbles. The tubes were freed from grease with nitric acid–chromic acid and well rinsed with water and the walls left wet. The resin suspension was poured into the accurately vertical tubes very carefully to avoid air being trapped. When this happened, the bubbles were immediately shifted by prodding with a piece of 20-gauge copper wire.

A small column, for preliminary separation of basic amino acids, had an overall length of 8 cm. and an internal diameter of 4·0 mm. It was cut off straight and a sintered disk (porosity 1) was sealed in flush with the bottom of the tube. The top had a B 7 socket with hooks. The tube was packed with resin, the total height of the resin being $2{\cdot}2 \pm 0{\cdot}1$ cm.

Fraction collector. A photoelectric drop-counting fraction collector (Locarte Co., 24 Emperors Gate, London, S.W. 7), capable of collecting 160 fractions in 6 in. × $\frac{5}{8}$in. test tubes, was used. Drops formed at the square-cut, lower end (external diam. 3·2 mm.) of a small glass funnel, attached by a Terry clip to a rigid brass plate, which itself was attached by similar clips to the two rods supporting the photocell. The position of the bottom of the funnel relative to the photocell was adjusted in two directions by sliding the clips, until the growth of the small drops reduced the

photocell current as far as possible below its standing, maximum, uninterrupted value. This adjustment was facilitated by a microammeter (full scale deflexion 500 μA and with shorting switch) permanently fitted in series with the photocell. Fractions of 0·2 ml. were obtained by collecting 8 drops of buffer containing detergent. The tip of the funnel was approx. 2 cm. above the top of the test tubes and was protected from draughts. Little evaporation from the fractions occurred overnight and no significant amount of ammonia was absorbed except in the neighbourhood of animal rooms. The funnel was kept permanently in the optimum position on the fraction collector and the blob at the bottom of the column brought gently into contact with the inside of the funnel at the beginning of an experiment.

Modifications to spectrophotometer. A Uvispek spectrophotometer (Hilger & Watts Ltd., model H 700.308) was temporarily modified as follows, so that readings could be made on 1·6 ml. of soln. in a standard 2 cm. cell. The cell was raised on a metal block 25 mm. × 13·5 mm. and 5·3 mm. in height, placed in the cell carrier. A diaphragm, consisting of a rectangular hole 4·5 mm. high and 1·8 mm. wide cut in a disk of black photographic paper, cemented to the end of a blackened metal tube (outside diam $\frac{7}{8}$ in. and inside diam. $\frac{1}{2}$ in. × 0·65 in. long), was inserted as close as possible to the exit slit, to obstruct the upper part of the light-beam. A slit width of 0·05 mm. and an input sensitivity of XI were used throughout with the meter sensitivity control set to position 4 for readings up to an extinction of 1·0, above which the meter sensitivity was increased as necessary.

METHODS

Operation of the columns

The columns were run as described by Eastoe (1955) except for the elution with pH 4·25 buffer being at 65° instead of 60°. After use, columns were cleaned with 0·2N-NaOH containing detergent. They were stored with alkali, the portion of the tube below the sinter being filled with water and closed with rubber tubing and clip while the top was stoppered. Where 1 mg. (or more) protein was available for analysis, separate (0·5 mg.) portions of hydrolysate were transferred to 100 and 15 cm. columns for determination of non-basic and basic amino acids respectively. Where less protein was available, a preliminary separation of basic amino acids was made on a column (2·2 cm. × 0·4 cm.) of Dowex 50. The hydrolysate in 0·2 ml. of citrate buffer, adjusted to pH 4·5–5·0, was added to this small column previously equilibrated with 0·1M-sodium citrate buffer, pH 5, and the eluate was collected in a conical graduated centrifuge tube. When all the hydrolysate had run into the resin, the column was eluted with more buffer, pH 5·0, in small (approx. 0·2 ml.) portions added as required. The elution was stopped when 1·3 ml. of eluate had collected. The eluate, which had been shown in preliminary experiments to contain all the acidic and neutral amino acids, was adjusted to pH 2·5 by adding 0·15 ml. of 2N-HCl, and transferred to the 100 cm. column after 5 days' storage at −15°, during part of which period analysis of the basic amino acids was carried out, there being only one fraction collector available.

Basic amino acids were quantitatively retained on the small column. When the hydrolysate does not contain hydroxylysine, the resin may be completely transferred by means of a Pasteur pipette to the top of a column (15 cm. × 0·4 cm.), previously equilibrated with buffer, pH 5. The elution is then carried out with the usual buffer sequence (Moore & Stein, 1951). Inversion and mixing of resin layers, resulting from the above operation, prevents the separation of hydroxylysine from histidine.

When hydroxylysine may be present, the small column is mounted above the 15 cm. column by means of a rubber bung (Fig. 1) and the two are

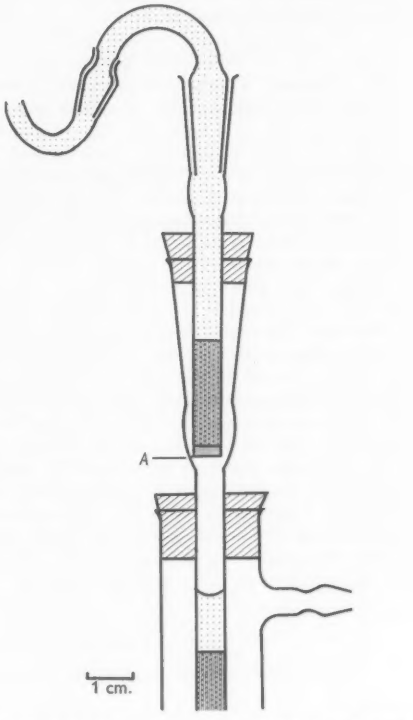

Fig. 1. Method of mounting a column (2·2 cm. × 0·4 cm.) of Dowex 50 above a column (15 cm. × 0·4 cm.) for elution of basic amino acids. Capillary contact is maintained at point *A* and free liquid remains above resin in lower column throughout the elution.

eluted as a single unit. The small column and bung should be inserted very carefully and slowly into the top of the 15 cm. column, to prevent increase of air pressure forcing the resin in the small column upwards, away from the sintered disk. One cm. of liquid should remain above the resin in the lower column to allow for rise of room temperature during the experiment. Otherwise the enclosed air may expand sufficiently for it to enter the resin below and stop the flow of liquid. Mixing, in the small volume of liquid between the two portions of resin, does not cause significant loss of resolution. Adequate separation of hydroxylysine and histidine with 0·1 M-sodium phosphate buffer, pH 6·75 (Eastoe, 1955), was obtained by this modification (Fig. 2).

It is necessary to elute for approx. 15% more fractions with each buffer to allow for the slight increase in the depth of resin. Early fractions, eluted with buffer, pH 5, should give no ninhydrin colour, serving to check the completeness of separation of non-basic amino acids.

Analysis of fractions

The volume of representative fractions was checked by weighing. All fractions were then adjusted to pH 5·0 by the addition of a definite number of drops of standard alkali or acid from calibrated jets, attached to glass reservoirs by short lengths of plastic tubing, each fitted with a screw clip. The volumes added are summarized in Table 1. Small corrections were made in the final calculations, where the fraction volume differed from 0·20 ml., and for the added drops. Portions (0·4 ml.) of ninhydrin solution (Moore & Stein, 1948) were added to the fractions from an automatic syringe pipette and the solutions were mixed by shaking the tubes. The tubes were covered with Oxoid aluminium caps (diam. $\frac{3}{4}$ in.) and heated for 20 min. in a boiling-water bath. The tubes were allowed to cool for 10 min., 1 ml. of ethanol–water (1:1, v/v) was added to each, and the solutions were mixed by shaking. The solutions were transferred to 2 or 0·5 cm. cells and the extinctions measured at 570 or 440 mμ in the spectrophotometer. Great care was necessary not to contaminate the solutions from the operator's fingers.

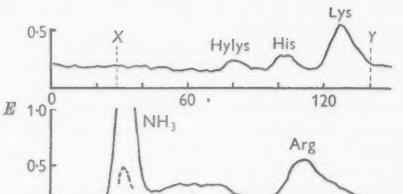

Fig. 2. Elution curve of the hydrolysate from 0·47 mg. of microsomal collagen, chromatographed on columns (2·2 cm. × 0·4 cm. and 15 cm. × 0·4 cm.) of Dowex 50 eluted as a single unit to separate the basic amino acids. The buffer was changed from 0·1 M-sodium citrate, pH 5, to 0·1 M-sodium phosphate, pH 6·75, at the point marked *X*, and finally to 0·2 M-sodium citrate, pH 6·5, at the point marked *Y*. Note the absence of neutral amino acids in fractions 5–20.

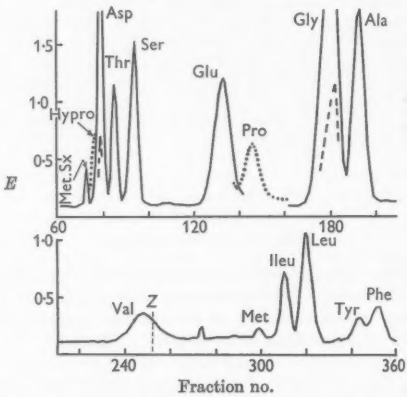

Fig. 3. Elution curve of the hydrolysate from 0·47 mg. of microsomal collagen, chromatographed on a column (100 cm. × 0·4 cm.) of Dowex 50 after initial separation of basic amino acids on a column (2·2 cm. × 0·4 cm.). —, $E_{570\ m\mu}$ with 2 cm. optical path; - - -, $E_{570\ m\mu}$ with 0·5 cm. optical path; . . . , $E_{440\ m\mu}$ with 2 cm. optical path for imino acids. The buffer was changed from pH 3·42 to pH 4·25 and the temperature from 37·5° to 65° at the point marked *Z*. Met.Sx, Methionine sulphoxide.

Table 1. *Volumes of solution required for neutralization of fractions*

All the buffers and both neutralizing solutions contained 0·4% (w/v) of BRIJ 35 to reduce surface tension.

Column	Buffer	Neutralizing soln.	Volume added (ml.)	No. of drops	Outside diam. of jet (in.)
100 cm. × 0·4 cm.	pH 3·42, 0·1 M-sodium citrate	N-NaOH	0·024	2	0·045
	pH 4·25, 0·1 M-sodium citrate	N-NaOH	0·012	1	0·045
15 cm. × 0·4 cm.	pH 6·75, 0·1 M-sodium phosphate	0·5 N-HCl	0·018	1	0·083
	pH 6·5, 0·2 M-sodium citrate	0·5 N-HCl	0·054	3	0·083

Determination of hydroxyproline. Hydroxyproline was determined by the method of Neuman & Logan (1950) as modified by Leach (1960). The determination was carried out in 6 in. × $\frac{5}{8}$ in. test tubes and the sample volume reduced from 1 ml. to 0·2 ml. with 1, 2 and 3 μg. standards. All reagent volumes were reduced to one-fifth of those specified, solutions being measured with automatic zero, blow-out micropipettes (H. J. Elliott Ltd.). Reproducibility was better than on the larger scale.

Resolution and accuracy of the semi-micro-chromatographic method

The ninhydrin colorimetric method (Moore & Stein, 1948) when tested with 0·2 ml. portions of standard glycine solution gave a mean calibration factor of 0·0380 μmole/unit of extinction for the 2 cm. light-path. This corresponded exactly with the value 0·190 previously obtained for 1 ml. fractions and a final volume of 8 ml. instead of 1·6 ml. The agreement suggested that colour formation was unaffected by the larger surface to volume ratio of the heated solutions on the smaller scale. Fading tests on the cold, diluted solutions gave erratic results but the fading rate averaged approx. 4%/hr., double that for the standard technique (Eastoe, 1955). It is therefore advisable to complete extinction measurements within 40 min. of the end of the heating period.

The resolution of the various peaks is illustrated in Figs. 2 and 3, showing the elution curve of the hydrolysate from 0·47 mg. of microsomal neutral-salt-soluble collagen on columns 0·4 cm. in diameter. This compares well with results obtained on columns 0·9 cm. in diameter with 5–10 mg. loadings (see Eastoe, 1955, Figs. 1 and 2). These results indicate that variation of the diameter of narrow columns does not greatly influence the positions and sharpness of peaks, and suggest that the diameter could be reduced if necessary below 0·4 cm.

Owing to the doubtful purity of some amino acids available from sources in this country (Bender *et al.* 1959), the accuracy of the semi-micro method was investigated by comparing results obtained for a standard protein hydrolysate (acid-processed pig-skin gelatin, hydrolysed with 6N-HCl for 48 hr. at 100°) by using columns of 0·9 and 0·4 cm. diameter (Table 2). On the whole the two methods show as close agreement as can be expected from the known errors of colorimetry, and the differences do not greatly exceed those of duplicate analyses on a single column 0·9 cm. in

Table 2. *Comparison of amino acid values for a hydrolysate of acid-processed, pig-skin gelatin with unmodified and semi-micro resin chromatographic methods*

Values are given as g. of amino acid/100 g. of dry ash-free protein. For abbreviations of amino acids see *Biochem. J.* (1957), **66**, 6.

Column diameter ...	0·9 cm.	0·4 cm.	
		Separate portions of hydrolysate on 100 and 15 cm. columns	Single portion separated on 2·2 cm. column
Ala	10·6	10·6	10·6
Gly	26·4	26·3	26·0
Val	3·24	3·01	3·25
Leu	3·36	3·50	3·54
Ileu	1·33	1·46	1·56
Pro	16·2	15·4	15·8
Phe	2·4	2·49	2·51
Tyr	0·51	0·46	0·45
Ser	3·89	4·09	3·86
Thr	2·27	2·32	2·24
Met	0·57	0·76	0·56
Arg	9·1	9·5	9·5
His	0·67	0·79	*
Lys	4·15	4·35	4·21
Asp	6·5	6·5	6·3
Glu	11·3	12·0	11·5
Hypro	12·7	(12·7)	(12·7)
Hylys	1·13	1·08	*
Total	116·32	117·31	115·96
Amide N	0·62	0·60	0·61
Wt. recovery (%)	97·2	98·0	97·0
N recovery (%)	101·5	102·5	101·4
Column loading (mg.)	5·8, 11·6	0·358 (×2)	0·358

* Sum of histidine and hydroxylysine 1·38.

diameter (Eastoe, 1955). The overall error with the semi-micro method is approximately ±5% for the larger peaks, compared with ±3% claimed for the original method (Moore & Stein, 1951). The increased error arises from the greater effect of variations in volume of test solutions and reagents together with fading. Errors for very small peaks may greatly exceed 5%, being mainly associated with assessing the base-line.

SUMMARY

1. A semi-micro modification of the Moore & Stein ion-exchange method of amino acid determination is described, which enables a complete analysis to be carried out on 0·3–0·6 mg. of protein.

2. The increased sensitivity obtained results from a reduction of resin-column diameter from 0·9 to 0·4 cm. with a corresponding reduction of the original volume of the fractions and reagents to one-fifth.

3. A simple method of modifying a spectrophotometer for convenient measurements on small volumes of solution in standard optical cells is described.

4. Preliminary separation of basic amino acids may be carried out on a column (2·2 cm. × 0·4 cm.) of Dowex 50 at pH 5·0. Separation of hydroxylysine from histidine is possible by combining the small column with a 15 cm. column and eluting the two in series.

5. Colorimetry on the reduced scale is closely similar to the original procedure, but the rate of fading is increased.

6. The sharpness and resolution of amino acid peaks were not affected by reduction of column diameter.

7. A comparison of amino acid values for a gelatin hydrolysate analysed by the original and semi-micro methods showed good agreement. The overall accuracy of the modified method is approximately 5%.

I should like to thank Mr J. H. Trendall for his help in making the chromatography tubes, and the British Empire Cancer Campaign for providing a spectrophotometer.

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Biochem. J. (1961) **79**, 656

Some Properties of Coupled Iron–Caeruloplasmin Oxidation Systems

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Caeruloplasmin, the blue copper-containing protein of plasma, has been reported to have oxidase activity *in vitro* towards many substrates. Holmberg & Laurell (1951) found activity towards *p*-phenylenediamine, quinol, catechol, pyrogallol, dihydroxyphenylalanine, adrenaline and ascorbic acid. They used purified caeruloplasmin in the presence of albumin or gelatin, which tend to eliminate any effects due to trace metals. Abood, Gibbs & Gibbs (1957), Porter, Titus, Sanders & Smith (1957), Martin, Eriksen & Benditt (1958), Sankar (1959), Nakajima (1959), Curzon & Vallet (1960) and Levine (1960) found that 5-hydroxytryptamine and some related hydroxyindoles were oxidized *in vitro* by caeruloplasmin. It is not known to what extent caeruloplasmin is involved in the metabolism of any of these substances *in vivo*.

During investigation of the effects of inorganic ions on the oxidase activity of purified human caeruloplasmin towards *NN*-dimethyl-*p*-phenylenediamine, it was found that the system was very sensitive to metals, both inhibition and activation being observed (Curzon, 1960). The most marked activating effect was found with ferrous iron. Curzon & O'Reilly (1960) showed that caeruloplasmin was able to oxidize ferrous to ferric iron and explained the activating effect of iron in terms of a coupled iron–caeruloplasmin oxidation system. This paper describes some properties of the system

and its effects on *NN*-dimethyl-*p*-phenylenediamine and on the substrates of physiological interest, 5-hydroxytryptamine, 5-hydroxyindolylacetic acid and adrenaline.

MATERIALS

A.R. grades of chemicals were used whenever obtainable. Purified human caeruloplasmin was prepared by the method of Curzon & Vallet (1960). A preparation with E_{605}/E_{280} 0·044 was used throughout except in the work described in Table 4, in which a less purified material with E_{605}/E_{280} 0·033 was used. Pure iron sponge was obtained from Johnson Matthey & Co. Ltd., London, E.C. 1. Stock ferric iron was prepared by dissolving 200 g. of ferric ammonium sulphate in 400 ml. of 5 mN-HCl and filtering. Ferrous iron solutions were freshly prepared from ferrous ammonium sulphate. 5-Hydroxytryptamine creatinine sulphate and 5-hydroxyindolylacetic acid were supplied by the Upjohn Co., Kalamazoo, Mich., U.S.A., adrenaline by British Drug Houses Ltd., Poole, Dorset, the human transferrin by Cutter Laboratories, Berkeley, Calif., U.S.A., and bovine plasma albumin by Armour Ltd., Eastbourne, Sussex. Other materials were as described by Curzon (1960). Stock reagents were stored in polythene bottles.

Purification of reagents. Since caeruloplasmin oxidation systems are very sensitive to traces of metal (Curzon, 1960), precautions were taken to avoid introduction of metal impurities. Acetic acid for preparing acetate buffers was distilled from glass and the fraction boiling between 126° and 130° collected. Sodium acetate (M) was extracted twice with 0·05 vol. portions of 0·01% of dithizone in CCl_4 by shaking occasionally during periods of 30 min. The first portion of dithizone became red-brown, indicating the presence of metal ions. The aqueous solution was then extracted four times with 0·05 vol. portions of CCl_4, twice with 0·05 vol. of 1% 8-hydroxyquinoline in $CHCl_3$, repeatedly with $CHCl_3$, then finally twice with CCl_4. 5-Hydroxytryptamine creatinine sulphate, 5-hydroxyindolylacetic acid and adrenaline (free base) were made up as 10 mM solutions in 0·2M-acetate buffer, pH 5·5, and similarly purified. Measurement of E_{278} before and after purification indicated that 4–5% of the 5-hydroxyindole material was lost during extraction procedures.

Buffers. 0·2M-Sodium acetate–acetic acid was used between pH 4·0 and 5·7. 0·2M-Sodium phosphate–sodium dihydrogen phosphate was used at pH 6·05 and 6·8.

METHODS

Apparatus was washed in Pyroneg detergent (Deosan Ltd., 42 Weymouth Street, London, W. 1) followed by water, 4N-HCl, water and deionized water. All solutions were made up in deionized water.

Determination of iron. The *o*-phenanthroline–hydroxylamine method described by Sandell (1959) was used. A primary iron standard was prepared by dissolving 500 mg. of iron sponge in 1·5 ml. of conc. H_2SO_4 and making to 1 l. with water.

Determination of oxidase activity of caeruloplasmin. Activity of caeruloplasmin towards *NN*-dimethyl-*p*-phenylenediamine dihydrochloride was measured under various conditions at 25° as previously described (Curzon, 1960). Activities are also given in terms of previously defined arbitrary units (Curzon & Vallet, 1960), of which 21 represent 1 μg. of caeruloplasmin copper.

Activities towards 5-hydroxyindoles were investigated as follows. To 2·0 ml. of various buffers (see below for details) were added 1·0 ml. of water or other additions and 1·0 ml. of 2 mM-5-hydroxytryptamine or -5-hydroxyindolylacetic acid. After incubation for 5 min. at 37°, 1·0 ml. of a suitable dilution of caeruloplasmin at 37° was added and incubation allowed to proceed at 37°. Identically treated blanks were run containing buffer, substrate and, where relevant, Fe^{2+} ions or ethylenediaminetetra-acetic acid (EDTA). At various times samples (1·0 ml.) were pipetted into conical centrifuge tubes and residual 5-hydroxyindole material was determined essentially as described by Udenfriend, Titus & Weissbach (1955) with the α-nitroso-β-naphthol colour reaction. The reagents were added rapidly to stop enzymic oxidation. The final purplish solution was centrifuged to remove denatured protein and the extinction was measured in a Unicam SP. 500 spectrophotometer with 1 cm. path microcells.

The shape of the spectral curve of the α-nitroso-β-naphthol colour obtained with the 5-hydroxyindolylacetic acid reaction mixture was identical with that obtained with pure 5-hydroxyindolylacetic acid. Residual 5-hydroxyindolylacetic acid was therefore determined from E_{540} as in the method of Udenfriend *et al.* (1955). Estimation of residual 5-hydroxyindolylacetic acid by paper chromatography gave results which agreed well with those obtained colorimetrically. The spectra obtained with 5-hydroxytryptamine digests were different from those obtained with the pure substance, and when E_{540} was used to determine residual 5-hydroxytryptamine results were much higher than when paper chromatography was used. This is because during digestion with caeruloplasmin, 5-hydroxytryptamine is oxidized to material which, on reaction with α-nitroso-β-naphthol, absorbs in the 400–600 mμ region, the absorption between 450 and 550 mμ being almost constant. The spectrum obtained with 5-hydroxytryptamine has a minimum at 450 mμ and a maximum at 546 mμ. When residual 5-hydroxytryptamine was determined from $E_{540} - E_{450}$ the results agreed well with those obtained by paper chromatography.

Activity of caeruloplasmin towards adrenaline was investigated as described above, except that 1·0 ml. of 2 mM-adrenaline was used as substrate. Adrenaline was oxidized to a pink pigment showing absorption maxima at 300 and 480 mμ characteristic of adrenochrome (Ellis & Jones, 1943). After various incubation times samples were cooled in ice–water and E_{300} was determined with 1 cm. microcells.

Paper chromatography. One-way ascending chromatograms of 5-hydroxytryptamine–caeruloplasmin and 5-hydroxyindolylacetic acid–caeruloplasmin reaction mixtures were run in the butan-1-ol–acetic acid–water system of Jepson (1955). The papers were sprayed with Ehrlich's reagent for indoles, and heated to develop colours as described by Curzon (1957). Residual 5-hydroxytryptamine and 5-hydroxyindolylacetic acid were estimated by comparison of spot areas with those of graded amounts of the relevant substances run in parallel. Brentamine fast red G.G. (British Drug Houses Ltd.) was also used as a reagent for phenolic groups (Harvey & Penketh, 1957).

RESULTS

Oxidations catalysed by the caeruloplasmin–iron system

NN-*Dimethyl*-p-*phenylenediamine.* When caeruloplasmin was added to a buffered solution of *NN*-dimethyl-*p*-phenylenediamine plus ferrous iron the oxidase activity as measured by the development of red colour was increased, as shown previously (Curzon & O'Reilly, 1960). The extinction at 550 mμ increased in an essentially linear manner with time of incubation, except with 0·1 mM-Fe^{2+} ion, which was the highest concentration of iron used (Fig. 1). If Fe^{2+} ion was preincubated with buffer plus caeruloplasmin at 25° before addition of *NN*-dimethyl-*p*-phenylenediamine, then, as the time of preincubation increased, the activation rapidly decreased (Table 1). This is probably due to the binding by various sites on the caeruloplasmin molecule of the Fe^{3+} ion formed. Under the standard conditions the *NN*-dimethyl-*p*-phenylenediamine reduces Fe^{3+} ion and protects the enzyme from the effects of this binding except at higher concentrations of Fe^{2+} ion.

When the concentration of added Fe^{2+} ion was kept constant and the activating effect measured at different concentrations of *NN*-dimethyl-*p*-phenylenediamine, percentage activation decreased as *NN*-dimethyl-*p*-phenylenediamine concentration increased (Table 2). Activation by Fe^{2+} ion was completely prevented by excess of EDTA, which also halved the activity in the absence of added iron, as shown previously (Curzon, 1960).

While 1 atom of Fe^{2+} ion/atom of caeruloplasmin copper causes reversible decolorizing of the caeruloplasmin during oxidation to Fe^{3+} ion (Curzon & O'Reilly, 1960), up to 10 atoms of Ni^{2+} or Co^{2+} ion/atom of caeruloplasmin copper did not cause decolorizing under identical conditions. Thus the definite, though relatively small, activation by these metals (Curzon, 1960) is apparently due to a different mechanism.

5-*Hydroxytryptamine.* The oxidation of 5-hydroxytryptamine at pH 5·5 is shown in Table 3. During incubation of 5-hydroxytryptamine with caeruloplasmin a yellow–brown colour developed. Paper chromatography showed that 5-hydroxytryptamine was destroyed and a new substance appeared, with R_F about 0·3, which gave a purple reaction with Ehrlich's reagent similar to that

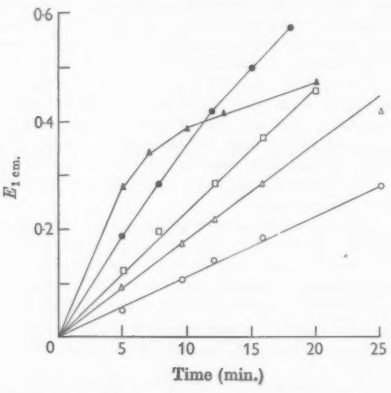


Fig. 1. Effect of ferrous iron on caeruloplasmin activity. The reaction mixture contained 0·08 M-acetate buffer, pH 5·5, 0·34 mM-*NN*-dimethyl-*p*-phenylenediamine dihydrochloride, various amounts of freshly prepared ferrous ammonium sulphate solution and 2·4 units of caeruloplasmin in a total volume of 5 ml. Incubation was at 25°; the reaction was stopped at various times by 2·0 ml. of 0·3 mM-sodium azide and $E_{1\,cm.}$ read at 550 mμ against identically treated blanks containing *NN*-dimethyl-*p*-phenylenediamine, buffer and relevant amounts of Fe^{2+} ion. ○, No Fe^{2+} ion added; △, 0·5 μM-Fe^{2+} ion; □, 2 μM-Fe^{2+} ion; ●, 10 μM-Fe^{2+} ion; ▲, 100 μM-Fe^{2+} ion.

Table 1. *Decrease of ferrous-iron activation by preincubation of ferrous iron with caeruloplasmin*

To 2·0 ml. of 0·2 M-acetate buffer, pH 5·5, at 25° were added 1 ml. of freshly prepared 0·05 mM-ferrous ammonium sulphate and 2·7 units of caeruloplasmin in 1 ml. of water at 25°. After incubation for various times, 1·0 ml. of 1·7 mM-*NN*-dimethyl-*p*-phenylenediamine held at 25° was added. After 15 min. the reaction was stopped by 2·0 ml. of 0·3 mM-sodium azide and $E_{1\,cm.}$ was read at 550 mμ. Activation by iron is expressed as percentage of *E* obtained when determinations were done by adding caeruloplasmin in buffer alone, preincubated as described above, to substrate + iron. Blanks were identically treated and contained *NN*-dimethyl-*p*-phenylenediamine and buffer and also Fe^{2+} ions when relevant.

Concn. of iron in reaction mixture (μM)	Percentage activation Preincubation times (min.)				
	0	3	15	30	60
10	220	155	31	−9	−4

Table 2. *Effect of ferrous iron on caeruloplasmin activity towards different concentrations of* NN-*dimethyl*-p-*phenylenediamine*

General conditions were as given in Fig. 1. Incubation time was 15 min.

Concn. of *NN*-dimethyl-*p*-phenylenediamine (mM)	Percentage increase of caeruloplasmin activity	
	0·38 μM-Fe^{2+} ion	1·0 μM-Fe^{2+} ion
0·67	66	146
1·0	58	137
2·0	48	95
4·0	33	83
16·0	15	53

Table 3. *Oxidation of 5-hydroxyindoles at 37° by caeruloplasmin and iron–caeruloplasmin systems*

Incubation and determinations were performed as described in the Methods section. Concentrations all refer to complete incubation mixture. 5HT, 5-Hydroxytryptamine creatinine sulphate; 5HIAA, 5-hydroxyindolylacetic acid.

Incubation mixture		5-Hydroxyindole destroyed (%) 30 min.	80 min.	170 min.
0·4 mM-5HT, pH 5·5; 8 units of caeruloplasmin	+ water	10	48	86
	+ 0·36 μM-Fe^{2+}	10	46	86
	+ 1·0 μM-Fe^{2+}	—	52	—
	+ 9·6 μM-Fe^{2+}	0	23	50
	+ 0·01 mM-EDTA	—	—	86
	+ 0·2 mM-EDTA	—	44	86
0·4 mM-5HIAA, pH 4·5; 8 units of caeruloplasmin	+ water	2	4	10
	+ 0·36 μM-Fe^{2+}	13	37	48
	+ 9·6 μM-Fe^{2+}	42	76	87
0·4 mM-5HIAA, pH 4·5; 32 units of caeruloplasmin	+ water	8	20	38
	+ 0·01 mM-EDTA	4	12	19
	+ 0·2 mM-EDTA	—	14	20

Table 4. *Effect of* pH *on oxidation of 5-hydroxyindoles by caeruloplasmin*

Incubation and determinations were performed as described in the Methods section. Buffers were all 0·08 M. Acetate buffers were used between pH 4·0 and pH 5·7 and phosphate buffers at pH 6·05 and 6·8. Incubation was for 90 min. at 37°. 5HT, 5-Hydroxytryptamine creatinine sulphate; 5HIAA, 5-hydroxyindolylacetic acid.

	5-Hydroxyindole destroyed (%)							
pH	4·0	4·55	4·70	5·2	5·5	5·7	6·05	6·8
0·4 mM-5HT, 0·2 mM-EDTA; 12 units of caeruloplasmin	0	8	—	64	77	76	26	0
0·4 mM-5HIAA, 0·2 mM-EDTA; 90 units of caeruloplasmin	24	27 (44)*	27	15	6	—	0	—

* Under these conditions some of the caeruloplasmin precipitated. Figure in parentheses is an estimate obtained by calculation from the results in Table 3.

given by 5-hydroxytryptamine itself. 5-Hydroxyindolylacetic acid and 5-hydroxytryptamine had R_F 0·82 and 0·58 respectively. Table 3 indicates that no evidence was obtained for the oxidation of 5-hydroxytryptamine by a coupled iron–caeruloplasmin system. Indeed, at 9·6 μM-Fe^{2+} ion some inhibition occurred. Also, in confirmation of the results obtained by H. Blaschko and W. G. Levine (personal communication), it was shown that destruction of 5-hydroxytryptamine was not significantly affected by EDTA. In agreement with Blum & Ling (1959) some oxidation of 5-hydroxytryptamine by Fe^{2+} ion was found. Chromatography of the product of oxidation by Fe^{3+} ion showed a complex mixture of substances giving a positive reaction with Erlich's reagent and which, like the products of oxidation of 5-hydroxytryptamine by Ag^{+} ion (Eriksen, Martin & Benditt, 1960), varied qualitatively in the presence of different anions.

5-Hydroxyindolylacetic acid. Preliminary paper-chromatographic experiments in which no special precautions were taken to avoid contamination by trace metals showed that the oxidation of 5-hydroxyindolylacetic acid by caeruloplasmin had an unusually low optimum pH of about 4·5 (Curzon & Vallet, 1960). Determination of 5-hydroxyindolylacetic acid destruction by caeruloplasmin in the presence of EDTA confirmed this (Table 4). During incubation with caeruloplasmin a very faint purple developed. It can be seen from Table 3 that oxidation of 5-hydroxyindolylacetic acid by caeruloplasmin, unlike that of 5-hydroxytryptamine, is extremely sensitive to iron, as little as 0·36 μM-Fe^{2+} ion causing a five- to eight-fold increase in activity. The oxidation of 5-hydroxyindolylacetic acid was unaffected by Fe^{2+} ion in the absence of caeruloplasmin. The activity of caeruloplasmin against 5-hydroxyindolylacetic acid is roughly halved in the presence of EDTA. Paper chromatograms of samples taken at various incubation times showed that as the 5-hydroxyindolylacetic acid spot at R_F 0·82 disappeared, a new substance appeared at R_F 0·62, giving a blue colour with Ehrlich's reagent and a weak yellow colour with diazotized Brentamine fast red G.G., which changed to a weak dirty purple on standing, unlike 5-hydroxyindolylacetic acid, which gave an im-

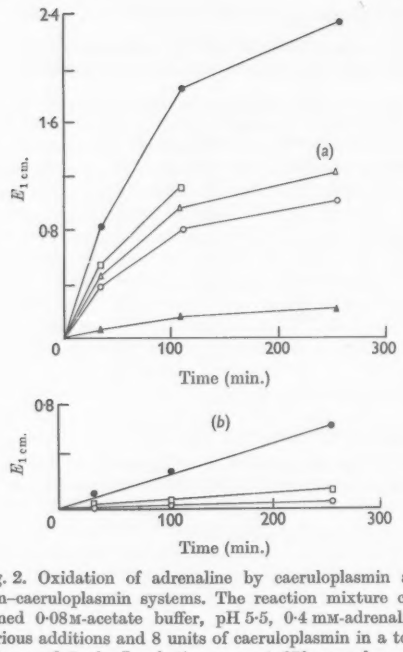


Fig. 2. Oxidation of adrenaline by caeruloplasmin and iron–caeruloplasmin systems. The reaction mixture contained 0·08M-acetate buffer, pH 5·5, 0·4 mM-adrenaline, various additions and 8 units of caeruloplasmin in a total volume of 5 ml. Incubation was at 37°, samples were removed at various times and cooled in ice–water and $E_{1\,cm.}$ was read at 300 mμ. ○, No additions; △, 0·36 μM-Fe^{2+} ion; □, 2 μM-Fe^{2+} ion; ●, 9·6 μM-Fe^{2+} ion; ▲, 200 μM-EDTA. In (*a*) results have been corrected for blank values obtained in the absence of caeruloplasmin. Some of these are shown in (*b*), the relevant symbols being used.

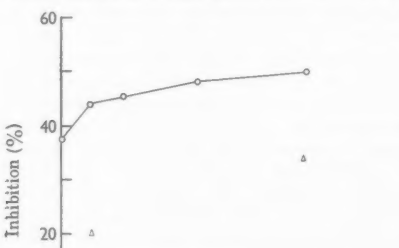


Fig. 3. Effect of protein on caeruloplasmin activity. General conditions were as given in Fig. 1. ○, Human transferrin; △, bovine plasma albumin. Caeruloplasmin present was 6·0 units; incubation time, 15 min.

mediate strong red. As incubation proceeded, the amount of oxidation product increased until it gave a spot on the chromatogram roughly equivalent in area to that of the original 5-hydroxyindolylacetic acid. Further incubation caused the spot to disappear gradually. The oxidation product was chromatographically identical with an indolic oxidation product previously produced in small yield when 5-hydroxyindolylacetic acid buffered at pH 7 was incubated in the presence of air (Curzon, 1957). The indolic oxidation product was also formed when 0·02M-5-hydroxyindolylacetic acid was oxidized at room temperature by an equal volume of ferric ammonium sulphate containing 1–2 g.atoms of Fe^{3+} ion/mole of 5-hydroxyindolylacetic acid. Higher concentrations of Fe^{3+} ion caused destruction of the indolic oxidation product.

Adrenaline–iron–caeruloplasmin system. The rate of oxidation of adrenaline by caeruloplasmin increased in the presence of Fe^{2+} ion and was much decreased, but not abolished, by EDTA (Fig. 2). Ferrous iron in the absence of caeruloplasmin caused some oxidation of adrenaline. Both a coupled iron–caeruloplasmin oxidation and autocatalysis by accumulated adrenochrome (Falk, 1949) are possible, though it is apparent from the shape of the oxidation–time curve that the latter is not dominant.

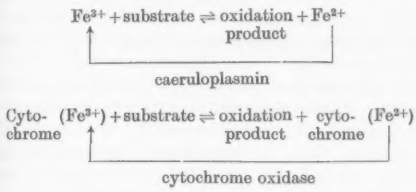
Further investigation of the oxidase activity of caeruloplasmin in the absence of added iron

Curzon (1960) found that EDTA partially inhibited the oxidation of *NN*-dimethyl-*p*-phenylenediamine by caeruloplasmin. Much evidence suggested that this was due to a direct effect of EDTA upon caeruloplasmin. On the other hand, the finding that oxidation of 5-hydroxytryptamine by caeruloplasmin was neither increased by Fe^{2+} ion nor decreased by EDTA suggests that EDTA inhibition of *NN*-dimethyl-*p*-phenylenediamine oxidation may involve chelation of activating trace metal and that in the absence of the latter EDTA would have been without effect. It has also been shown (Fig. 3) that transferrin partially inhibited activity against *NN*-dimethyl-*p*-phenylenediamine and that the curve of inhibition against log concentration of inhibitor was similar to that found previously with EDTA. Bovine plasma albumin inhibited more weakly. Transferrin also strongly inhibited the effect of added iron.

DISCUSSION

It has been known for many years that cupric copper catalyses the oxidation of ferrous iron (Lamb & Elder, 1931), and it is of interest that caeruloplasmin, the plasma copper–protein, can also catalyse this reaction. There is a formal re-

semblance between the caeruloplasmin–iron and cytochrome–cytochrome-oxidase systems which is strengthened by recent evidence for the involvement of copper in the enzymic function of cytochrome oxidase (Sands & Beinert, 1959) and by the fact that *p*-phenylenediamine and its derivatives are substrates of both systems.


Fe2+ + substrate ⇌ oxidation product + Fe2+
caeruloplasmin
Cyto-chrome (Fe2+) + substrate ⇌ oxidation product + cyto-chrome (Fe2+)
cytochrome oxidase

While the function of the cytochrome system is well established, that of caeruloplasmin is unknown. Iron is largely absorbed from the gut in the ferrous form (Moore, Arrowsmith, Welch & Minnich, 1939) and is oxidized to ferric iron before combination with the transferrin of plasma (Koechlin, 1952). The finding that the considerable iron-oxidizing power of blood (Starkenstein & Harvalik, 1933) is unaffected by cyanide (Hendrych & Mori, 1933), which inhibits caeruloplasmin, suggests that the latter is unlikely to play much part in this oxidation. On the other hand, Chase, Gubler, Cartwright & Wintrobe (1952) have found that copper-deficient rats absorb less iron than do rats supplied with copper. S. O'Reilly (personal communication) showed that coupled iron–caeruloplasmin oxidation may be demonstrated in the presence of plasma but at higher iron concentrations than those effective with purified caeruloplasmin.

Sensitivity of the oxidase activity of caeruloplasmin to iron is markedly dependent on the nature and concentration of the substrate used. Ferrous iron is essentially a second substrate in competition with the first substrate for the active centre of the enzyme. Thus variation of iron activation with *NN*-dimethyl-*p*-phenylenediamine concentration (Table 2) is explicable in terms of substrates competing for the enzyme, although it is also possible that competition between *NN*-dimethyl-*p*-phenylenediamine and caeruloplasmin for iron may be involved since iron–*p*-phenylenediamine complex formation has been demonstrated (Hara, 1951). Any substance which is oxidizable by ferric iron is potentially oxidizable by the coupled iron–caeruloplasmin system. The effectiveness of this system and its ability to compete with a direct caeruloplasmin–substrate interaction vary widely from substrate to substrate.

Martin *et al.* (1958) found that plasma has no oxidase activity against 5-hydroxyindolylacetic acid at pH 6·0. Caeruloplasmin at pH 4·5 has small but definite activity in the absence of added iron which is about half inhibited by 0·01–0·20 mM-EDTA. The rather acid optimum, pH 4·5, is of interest as an optimum pH 5·5 has been established for many substrates (Holmberg & Laurell, 1951), and also in the present study for 5-hydroxytryptamine. Aprison, Hanson & Austin (1959) showed that, unlike 5-hydroxytryptamine, which was a competitive inhibitor, 5-hydroxyindolylacetic acid inhibited the activity of caeruloplasmin against *NN*-dimethyl-*p*-phenylenediamine non-competitively at pH 6·2 and suggested that the ionized carboxyl group of the acid was involved in the inhibited complex. This is in agreement with the finding that 5-hydroxytryptamine but not 5-hydroxyindolylacetic acid is a substrate at pH 6·05. The markedly acid optimum pH for 5-hydroxyindolylacetic acid breakdown is explicable in terms of two caeruloplasmin–5-hydroxyindolylacetic acid interactions, formation of an enzyme–inhibitor complex involving the ionized carboxyl and also of an enzyme–substrate complex. Results suggest that at pH 6·05 the former entirely predominates but at more acid pH some depression of carboxyl ionization occurs and a slight oxidase activity is manifested.

5-Hydroxyindoles may give rise to indolic autoxidation products (Curzon, 1957). The autoxidation product formed from 5-hydroxyindolylacetic acid has been found in the urine of subjects with carcinoid disease (Dalgliesh, 1956; Curzon, 1957), although it is not clear whether it is a metabolite or an artifact: certainly it tends to increase on storage of the urine. The substance behaves chromatographically like the unidentified indole U2 found by Jepson (1955) in urine of subjects with carcinoid disease. Formation of apparently identical material by oxidation of 5-hydroxyindolylacetic acid with caeruloplasmin and of the mixture of indolic oxidation products obtained by oxidation of 5-hydroxytryptamine with caeruloplasmin, in agreement with Eriksen *et al.* (1960), is of interest as it suggests a possible pathway of metabolism of 5-hydroxyindoles which could lead to a hitherto unknown series of indolic substances. As has been pointed out by these authors, there are many structural possibilities for ring-oxidation products of 5-hydroxyindoles. They give evidence supporting formation of one of the 5-hydroxytryptamine oxidation products by oxidative dimerization. Even much simpler aromatic hydroxy compounds may be oxidized to quite complex substances, e.g. the oxidation by ferricyanide of *p*-cresol to a tricyclic dimerization product analogous to usnic acid (Barton, Deflorin & Edwards, 1956) and containing an aromatic keto group but no free phenolic groups. The weak atypical phenolic reactions shown by both the 5-hydroxyindolyl-

acetic acid oxidation product and by the dimeric 5-hydroxytryptamine oxidation product (Eriksen *et al.* 1960) suggest that perhaps the reactions by which they are formed may be to some extent analogous to the *p*-cresol oxidation.

Interpretation of the partial inhibition by EDTA of *NN*-dimethyl-*p*-phenylenediamine oxidation by caeruloplasmin (Curzon, 1960) presents considerable difficulty. The effect may reasonably be considered as due either to chelation of activating contaminant metal or to a direct interaction between caeruloplasmin copper and EDTA. The first explanation is consistent with the great enhancement of *NN*-dimethyl-*p*-phenylenediamine oxidation by iron, which is inhibited by EDTA, by the lack of effect of both iron and EDTA on the oxidation of 5-hydroxytryptamine by caeruloplasmin and also by the partial inhibition of the *NN*-dimethyl-*p*-phenylenediamine oxidation by transferrin. Use of transferrin for investigation of the significance of iron in enzyme systems has, however, been criticized by Singer & Massey (1957) because of anomalous results obtained with succinic dehydrogenase.

A direct interaction between caeruloplasmin and EDTA is consistent with the lack of effect on inhibition by EDTA of all procedures for the removal of contaminant metal from the system and by the undetectability of significant contaminant metal in the system (Curzon, 1960). In addition, Humoller, Mockler, Holthaus & Mahler (1960) find that EDTA gives 50% inhibition of *NN*-dimethyl-*p*-phenylenediamine oxidation by caeruloplasmin in 0·66M-acetate buffer, pH 6·0, though at the much higher EDTA concentration of 1·5 mM. This corresponds exactly to the discontinuity in the percentage inhibition–log EDTA concentration curve demonstrated by Curzon (1960). This exact quantitative agreement between two independent series of experiments in which different conditions and materials were used suggests that a direct interaction between caeruloplasmin and EDTA may be involved rather than contamination by trace metals. The evidence for 50% accessibility of caeruloplasmin copper to EDTA is paralleled by its 50% exchangeability with ^{64}Cu (Scheinberg & Morell, 1957) and its 50% dialysability after chymotryptic digestion (Curzon, 1958).

The uncertainties inherent in the interpretation of the effect of a metal-binding agent on an enzyme which contains metal bound in two different ways and which is also affected by traces of extraneous metal are, however, considerable and caution is necessary in the interpretation of results.

SUMMARY

1. Some properties of coupled iron–caeruloplasmin oxidation systems have been investigated.

2. Ferrous iron enhances the activity of caeruloplasmin against *NN*-dimethyl-*p*-phenylenediamine, the effect decreasing with increasing *NN*-dimethyl-*p*-phenylenediamine concentration and upon preincubation of iron with caeruloplasmin. Enhancement is prevented by ethylenediaminetetra-acetic acid or excess of protein.

3. 5-Hydroxytryptamine and 5-hydroxyindolylacetic acid are oxidized to indolic products by caeruloplasmin, the optima being about pH 5·5 and 4·5 respectively.

4. Oxidation of 5-hydroxytryptamine by caeruloplasmin is neither inhibited by ethylenediaminetetra-acetic acid nor enhanced by iron under the conditions used. Oxidation of 5-hydroxyindolylacetic acid is partly inhibited by ethylenediaminetetra-acetic acid and greatly enhanced by iron.

5. Oxidation of adrenaline to adrenochrome by caeruloplasmin is partly inhibited by ethylenediaminetetra-acetic acid and is enhanced by iron.

6. The inhibition of caeruloplasmin by ethylenediaminetetra-acetic acid is discussed in the light of these and of earlier findings.

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Inactivation of Arginine Vasopressin by Rat-Kidney Slices

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Previous studies have indicated that the mammalian kidney can inactivate as well as excrete vasopressin. Only 5–10% of a dose of vasopressin injected intravenously into rats can be recovered from the urine (Heller, 1952; Ginsburg, 1954), although the kidneys have been found responsible for removing approximately half of the injected hormone from the circulating blood (Ginsburg & Heller, 1953; Dicker, 1954; Crawford & Pinkham, 1954; Lauson & Bocanegra, 1960). There is suggestive evidence *in vivo* that this unexcreted vasopressin is destroyed by the kidney (Heller & Zaidi, 1957). At the present time, however, little is known about the nature of the cellular or enzymic processes involved in this inactivation of vasopressin. In the present paper we have attempted *in vitro* to develop a system useful for such investigations. Also reported are studies in which vasopressin, labelled *in vivo* with [^{35}S]cystine, has been used in an effort to study the manner in which this polypeptide hormone is inactivated by rat-kidney slices.

EXPERIMENTAL

Materials. Arginine vasopressin was purified as described by Sachs (1960*a*) with an estimated potency of 300–400 i.u./mg. This material showed only a single component when subjected to paper ionophoresis (Sachs, 1960*a*) or paper chromatography (Heller & Lederis, 1958). Oxytocin was highly purified synthetic oxytocin with a stated potency of 450 i.u./ml. from Sandoz Co., Hanover, N.J., U.S.A. Heparin was from Upjohn Co., Kalamazoo, Mich., U.S.A. Phenoxybenzamine hydrochloride (Dibenzyline) was from Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A.

Preparation of kidney slices. Male Sprague–Dawley rats weighing from 200 to 350 g. were stunned by a blow on the head and killed by decapitation. A midline incision was made and both kidneys, freed from perirenal fat and decapsulated, were placed in ice-cold Cross & Taggart (1950) buffer containing 0·01 M-sodium acetate. Longitudinal sections of 0·5 mm. thickness were cut with a Stadie–Riggs microtome; the first slice on each side of the kidney was rejected and the second slice placed in fresh ice-cold buffer. Each slice weighed about 75 mg. and contained both cortical and medullary tissue. These operations were performed at 6°.

Incubation procedure. Unless otherwise stated, each incubation flask contained four kidney slices in 4 ml. of Cross & Taggart buffer (containing about 300 mg. of tissue). Slices and buffer were initially shaken for 30 min. at 25° under O_2, after which time the total wet weight of the slices was determined and the slices were transferred to 4 ml. of fresh buffer. Vasopressin (3 i.u.) was added and the incubation continued, under the same conditions, for a further time interval ranging from 30 to 120 min. During this second incubation period, measured portions of the incubation fluid were periodically taken for assay; the samples were pipetted into 5% trichloroacetic acid to stop any enzymic reaction. The trichloroacetic acid was subsequently removed by ether extraction (4 × 3 vol.). Ether dissolved in the aqueous phase was evaporated in a stream of N_2 at 40°, and the samples were assayed for pressor activity.

Assay of pressor activity. The method of Dekanski (1952) was used except that Dibenzyline (1 mg./100 g. body wt.) was injected intramuscularly 12–24 hr. before the beginning of assay. Anaesthesia was induced by intraperitoneal

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injection of a 25% (w/v) solution of urethane (0·4 ml./100 g. body wt.). A (2 by 2) dose assay was used in duplicate on all occasions.

Preparation of [^{35}S]*vasopressin.* [^{35}S]Cystine was prepared as described by Sachs (1960*a*) and purified by chromatography on Dowex 50 (Hirs, Moore & Stein, 1954). The purified [^{35}S]cystine was treated with thioglycollic acid, and subsequently 2×10^8 counts/min. of the reduced material were infused continuously into the third ventricle of an anaesthetized, 15 kg. male dog, over a period of 8 hr. After the infusion was complete, the animal was killed and the hypothalamus and neurohypophysis were excised, combined and used for the isolation of [^{35}S]vasopressin as described by Sachs (1960*b*).

The purification steps included: (1) homogenization of the tissue in 0·1 N-acetic acid; (2) precipitation of proteins with 10% (w/v) trichloroacetic acid; (3) addition of 300 i.u. of highly purified carrier arginine vasopressin to the trichloroacetic acid-soluble extract (which contained 18 i.u. of pressor activity); (4) removal of trichloroacetic acid with ether and passage of the aqueous extract through a small Dowex XE-64 resin (H form); (5) elution of the hormone with pyridine–acetate, and, after removal of solvent *in vacuo*, chromatography of vasopressin on a carboxymethylcellulose ion-exchange column (0·9 cm. × 30 cm.); (6) rechromatography of the biologically active peak on Dowex XE-64 (ammonium form) with a continuous-gradient elution procedure; and finally (7) adsorption and elution of the active peak on a small (2·0 cm. × 1·0 cm.) column of Dowex XE-64 (H form). The amount of [^{35}S]-vasopressin obtained was approx. 123 i.u. and contained a total of 370 counts/min. Previous experiments (Sachs, 1960*a*, *b*) have shown that [^{35}S]vasopressin obtained at this stage of purification was essentially pure.

Radioactive materials were plated at infinite thinness on stainless-steel planchets, and counting was performed with a thin window Geiger–Müller tube and scaler (Nuclear Instrument and Chemical Corp., Chicago, Ill., U.S.A.).

Buffers. Those used were: citrate–phosphate buffer (McIlvaine, 1921), consisting of 0·1 M-citric acid mixed with 0·2 M-disodium hydrogen phosphate; 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (Michaelis, 1930), consisting of 0·2 M-tris mixed with 0·2 M-hydrochloric acid; borax–sodium hydroxide buffer (Clark & Lubs, 1948), consisting of 0·05 M-borax mixed with 0·2 M-sodium hydroxide.

RESULTS

Recovery and estimation of vasopressin from incubation media. It was first necessary to determine whether vasopressin was stable in Cross–Taggart buffer and to determine the percentage of vasopressin which was lost due to transference of samples to trichloroacetic acid and subsequent ether extraction. Accordingly, 3 i.u. of vasopressin in 4 ml. of Cross–Taggart buffer was incubated and samples were transferred to trichloroacetic acid at the beginning, and after 60 min., of incubation at 25°. Half of the samples contained bovine serum albumin (2·5 mg./ml.) in addition to vasopressin, to ascertain whether the presence of protein would lower the efficiency of the extraction procedure. No difference in recovery could be detected between any of the samples. The recovery of vasopressin from 16 experiments was 88·9 ± 2·9% (mean ± S.D.), and all future vasopressin determinations were corrected on the basis of this recovery.

Time course of vasopressin disappearance during incubation with rat-kidney slices. The rate of disappearance of vasopressin from the incubation medium in the presence of kidney slices (approx. 75 mg./ml.) was studied under the standard conditions (see Experimental section). The rate of disappearance of the hormone from the medium essentially follows a first-order reaction (Fig. 1). An apparent first-order velocity constant was calculated for each of four such experiments. These apparent first-order rate constants have been corrected to represent those obtained from 75 mg. of tissue/ml. of incubation medium and have been given the symbol k_{75}; the mean k_{75} value equalled 0·0109 ± 0·0005 min.$^{-1}$. The validity of the correction applied for kidney-slice wt./ml. of incubation medium is demonstrated below.

Necessity for the presence of the kidney slice during vasopressin disappearance. In order to exclude the possibility that soluble enzymes liberated into the incubation medium by the kidney slice were responsible for inactivation of vasopressin, the following control experiments were performed. Kidney slices (approx. 75 mg./ml.) were incubated under standard conditions for 60 min. in the absence of vasopressin. The slices were removed from the medium, vasopressin was added (0·75 i.u./ml.), and the incubation was continued for a further

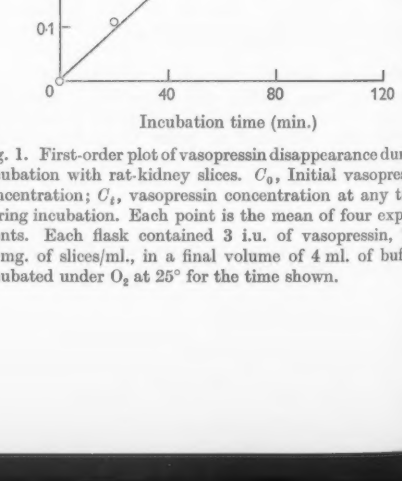

Fig. 1. First-order plot of vasopressin disappearance during incubation with rat-kidney slices. C_0, Initial vasopressin concentration; C_t, vasopressin concentration at any time during incubation. Each point is the mean of four experiments. Each flask contained 3 i.u. of vasopressin, 70–80 mg. of slices/ml., in a final volume of 4 ml. of buffer, incubated under O_2 at 25° for the time shown.

60 min., during which time samples were taken for assay at 20 min. intervals. Less than 10% of the added vasopressin was lost during this 60 min. period, whereas, when slices were present, over 50% of the added vasopressin was lost during the first 60 min. incubation. Soluble enzymes prepared by incubating kidney slices at 37° (instead of 25°), which were capable of inactivating vasopressin, were shown to be stable under the standard incubation conditions.

Effect of various amounts of kidney tissue on the rate of vasopressin disappearance. The disappearance of vasopressin incubated under standard conditions in the presence of various amounts of kidney slices was determined over a period of 2 hr. The apparent first-order rate constant was calculated for the different amounts of kidney tissue used. Fig. 2 shows a linear relationship between the apparent rate constant and the amount of kidney tissue used throughout the range studied, 0–150 mg. of kidney slice/ml. Unless otherwise stated, subsequent experiments employed 280–320 mg. of tissue in the incubation medium. The rates of hormone disappearance were corrected to that expected from using 300 mg. of kidney tissue.

Effect of vasopressin concentration on the rate of vasopressin disappearance. The rate of vasopressin disappearance was measured in a series of experiments where various concentrations of hormone (0·45–15·3 i.u./ml., or approx. 1·04–35·3 μM respectively) were incubated with slices under standard conditions. In Table 1 are shown the k_{75} values calculated for each vasopressin concentration.

At low vasopressin concentrations (0·5–2·0 i.u./ml.), the apparent rate constant remained unchanged, but as the concentration of hormone was increased above 3 i.u./ml., the k_{75} values fell progressively. A Lineweaver–Burk plot gave half-maximal velocity at a vasopressin concentration of 23·8 i.u./ml. (or 55 μM, assuming that 1 mg. of vasopressin is equivalent to approx. 400 i.u.).

Unless stated otherwise, the standard incubation flask contained an initial vasopressin concentration of 0·75 i.u./ml. (1·7 μM). This choice of vasopressin concentration had the advantage of being within the substrate concentration range (0·5–2·0 i.u./ml.) where the apparent rate constant was unchanging and maximal. Also, greatest percentage loss of vasopressin occurred under these conditions, so enabling high accuracy in the determination of k_{75} values.

Effect of pH *on the loss of vasopressin during incubation with rat-kidney slices.* The medium used (Cross & Taggart) for the incubation of slices and vasopressin contained 8 mM-phosphate buffer. The initial pH of the complete standard system was 7·15 and remained unaltered throughout an experiment. At more acid or alkaline pH values, it was necessary to increase the buffer strength to 0·04 M to maintain a constant pH during the course of the incubation. Increasing the buffer strength from 8 mM to 0·04 M at pH 7·15 did not affect the rate of disappearance of vasopressin. In addition, replacing the phosphate buffer with either citrate–phosphate or tris buffer (both at pH 7·15) did not affect the rate of loss of vasopressin from the incubation medium. However, at pH 4·0 and pH 5·5 (citrate–phosphate) little or no loss of vasopressin occurred after 40 min. incubation in the presence of slices. On the other hand, at pH 7

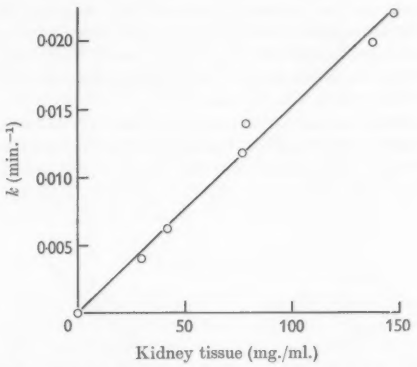

Fig. 2. Effect of changing the weight of kidney tissue on the apparent first-order rate constant. The apparent rate constant (k) is calculated from the slope of a plot as shown in Fig. 1; the slope was the best straight line calculated from four experimental points. Each flask contained 3 i.u. of vasopressin and 2–8 kidney slices in 4 ml. of buffer, incubated under O_2 at 25° for 120 min. Samples were taken for assay at 40 min. intervals.

Table 1. *Effect of vasopressin concentration on the k_{75} values*

Each flask contained four kidney slices and various amounts of vasopressin in 2 ml. of Cross & Taggart buffer and was incubated under O_2 at 25° for 60 min. k_{75} values were calculated from three experimental points. i.u./ml. $\times 2{\cdot}307 \times 10^{-3}$ = μmoles/ml.; this is based on the assumption that each mg. of vasopressin is equivalent to 400 i.u.

Initial vasopressin concn. (i.u./ml.)	0·45	0·89	1·88	3·23	5·79	11·3	12·7	15·3
$10^2 k_{75}$ (min.$^{-1}$)	1·03	0·98	1·06	0·93	0·75	0·57	0·41	0·31

and 8 (phosphate or tris buffer) or pH 9 and 10 (borax–sodium hydroxide buffer), from one-third to one-half of the vasopressin was lost from the incubation fluid after 40 min. of incubation. There appeared to be no significant difference in the amount of vasopressin lost over the pH range 7–10. Control experiments showed that the loss of vasopressin at the higher pH values (pH 9 and 10) was not due to soluble enzymes liberated into the incubation medium; negligible quantities of hormone were lost after 40 min. of incubation with medium (pH 9 or 10) previously incubated with slices alone.

Effect of temperature on the rate of vasopressin disappearance from the incubation medium. Under the standard incubation conditions at 25°, k_{75} for vasopressin disappearance was consistently reproducible: 0·0109 ± 0·0005 min.$^{-1}$ (four experiments). Fig. 3 shows that at 7° the rate of vasopressin loss, though still first-order, was much slower. The mean k_{75}, calculated from four experiments, was 0·0026 ± 0·004 min.$^{-1}$, which was significantly different from the k_{75} determined at 25°. The Q_{10} (that is the factor by which the apparent velocity constant is altered by a 10° change in temperature) calculated from the two mean k_{75} values was approx. 2·0. Control experiments in which vasopressin (0·75 i.u./ml.) was incubated for 60 min. with buffer previously incubated at 7° with kidney slices showed no detectable loss of vasopressin.

When kidney slices were incubated with vasopressin under standard conditions at 37° (instead of 25°), the pressor activity disappeared rapidly from the incubation medium during the first 60 min. However, it could be shown that slices incubated at 37° for 60 min. (instead of at 25°) liberated heat-labile (100°; 2 min.) material into the incubation medium capable of inactivating vasopressin. Such media inactivated as much as 66 % of added vasopressin over a period of 60 min. at 25°.

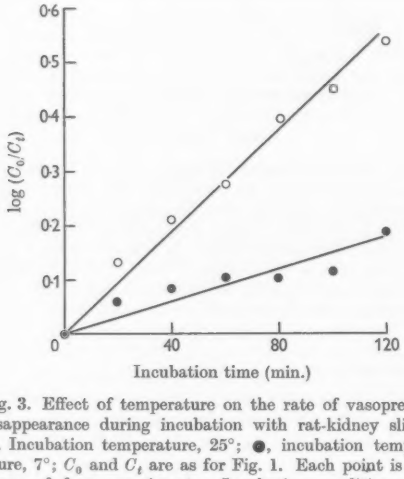

Fig. 3. Effect of temperature on the rate of vasopressin disappearance during incubation with rat-kidney slices. ○, Incubation temperature, 25°; ●, incubation temperature, 7°; C_0 and C_t are as for Fig. 1. Each point is the mean of four experiments. Incubation conditions are given in the legend to Fig. 1.

Effect of inhibitors on vasopressin loss from the incubation medium. A number of substances, which have been previously shown to inhibit either the energy-yielding and transport mechanisms of kidney cells or the activity of a variety of proteolytic enzymes, were tested for their ability to inhibit the inactivation of vasopressin by kidney slices.

Dinitrophenol at a concentration of either 0·1 or 1 mM (in the presence or absence of acetate) did not affect the rate of loss of vasopressin from the incubation medium; 1 mM-*p*-chloromercuribenzoate gave a 30 % inhibition; 1 mM-di*iso*propyl phosphorofluoridate (preincubated with slices), soya-bean trypsin inhibitor (1 mg./ml.), 0·04 M-ethylenediaminetetra-acetate and 0·2 M-phosphate (pH 7·15), did not appear to affect the rate of loss of vasopressin. On the other hand, oxytocin, a peptide with a structure similar to vasopressin, showed a marked ability to inhibit the rate of loss of vasopressin from the incubation medium. Under standard conditions (0·75 i.u. of vasopressin/ml.), the addition of synthetic oxytocin (15 i.u./ml.) inhibited the rate of loss of vasopressin by about 40–50 %. The mean k_{75} for vasopressin loss under these conditions was 0·0059 ± 0·00025 min.$^{-1}$ (four experiments). If oxytocin were destroyed when incubated with vasopressin in the presence of kidney slices the inhibition of vasopressin destruction would be even greater since, for each removed portion of the incubation medium assayed for vasopressin, a constant correction had been made for the pressor activity of oxytocin, assuming each i.u. of oxytocin had 0·95 % of the pressor activity of an i.u. of vasopressin.

Fate of vasopressin incubated with kidney slices

Experiments were carried out to determine whether vasopressin which disappeared from the incubation medium was taken up and stored by the kidney slice. Parallel incubations were performed under standard conditions in flasks containing either the complete standard system or slices and buffer with no added vasopressin. After 60 min. at 25°, samples were taken from the incubation media (in order to determine the amount of hormone lost) and the slices were simultaneously removed from the incubation fluid and homogenized with 10 % (w/v) trichloroacetic acid. The homo-

genates were centrifuged and the precipitated proteins washed several times with 5% (w/v) trichloroacetic acid; the washings were combined with the first trichloroacetic acid supernatant. Solutions were treated with ether in order to remove trichloroacetic acid, then the aqueous extracts were assayed for pressor activity. Both the control (slices incubated alone) and test (slices incubated with vasopressin) extracts contained barely detectable amounts of pressor activity, 25·3 and 27·8 milliunits/300 mg. of kidney tissue respectively (mean of three experiments). These figures have been corrected for vasopressin recovery of 72·7%, which was the mean recovery (three experiments) in which kidney slices were homogenized with trichloroacetic acid in the presence of known quantities of vasopressin, and the extracts treated as described above.

The small amount of pressor activity (which probably was not vasopressin) recovered from kidney slices incubated with vasopressin would account for only 2·2% of the hormone lost during the 60 min. incubation period. Apparently then, vasopressin, incubated with kidney slices, was transformed into some inactive product (or products).

Quantitative ninhydrin determinations on paper and ion-exchange chromatography of the incubation fluid (before and after incubation) failed to detect the presence of newly formed amino groups or peptide material resulting from the inactivation of vasopressin. However, this inability to demonstrate any peptide- (or amide-) bond splitting of vasopressin was in all likelihood due to the presence of relatively large quantities of ninhydrin-reactive material arising from the kidney slice. Experiments were therefore initiated with [^{35}S]-vasopressin as substrate, in order (1) to confirm the biological recovery experiments, and (2) to obtain some information about the chemical properties of the inactivation product or products. Accordingly, [^{35}S]vasopressin (123 i.u.; 370 counts/min.) was incubated in 5·3 ml. of the standard buffer which contained 1·5 g. of kidney slices. Before incubation, a zero-time sample (30 i.u.; 91 counts/min.) was removed and added to 2 vol. of 15% (w/v) trichloroacetic acid. After 30 min. at 25° a measured portion was removed from the incubation medium for assay in the usual manner; the remainder of the incubation fluid was poured into 10 ml. of 15% (w/v) trichloroacetic acid. The slices were separately treated with trichloroacetic acid as described above. All the trichloroacetic acid supernatant solutions and the washings were combined, extracted with ether and adjusted to pH 5·0 with dilute sodium hydrogen carbonate. The zero-time sample was treated in a similar manner. These two aqueous extracts (obtained at zero time and after 30 min. of incubation) were each passed through a Dowex XE-64 (H form) ion-exchange column (0·9 cm. × 2·0 cm.). Acetic acid [50 ml. of 0·25% (v/v)] was passed through the column, followed by 40 ml. of pyridine–acetic acid solution [30% (v/v) pyridine, 4% (v/v) acetic acid] and finally 25 ml. of 0·2N-hydrochloric acid. Each of the effluent fractions was assayed for vasopressin and radioactivity. The results are: (*a*) amount of [^{35}S]vasopressin removed at zero time, 30 i.u., 91 counts/min.; (*b*) amount of vasopressin in the zero-time sample eluted from Dowex XE-64 with pyridine–acetic acid, 21 i.u., 70 counts/min.; (*c*) amount of [^{35}S]vasopressin incubated with kidney slices, 93 i.u., 279 counts/min.; (*d*) amount of vasopressin activity remaining after 30 min., 60 i.u.; (*e*) amount inactivated, 33 i.u.; (*f*) amount of vasopressin eluted from Dowex XE-64 with pyridine–acetic acid, 44 i.u., 184 counts/min.

The other effluent fractions did not contain either vasopressin activity or radioactivity.

In addition there were no detectable counts above background (13·7 counts/min.) associated with the kidney-slice proteins, which were precipitated and washed with trichloroacetic acid and then ether, plated, and counted.

Apparently all the recovered counts which included [^{35}S]vasopressin and ^{35}S-labelled inactivation products were adsorbed on Dowex XE-64 (a cation-exchange resin) at pH 5·0 and could be eluted with pyridine–acetic acid solution. The zero-time sample gave identical results except that the specific activity of the recovered [^{35}S]vasopressin remained essentially unchanged from the specific activity of the original starting material.

DISCUSSION

Both crude and partially purified fractions of mammalian kidney extracts (Heller, 1952; Dicker, 1960) have been shown to be capable of inactivating vasopressin *in vitro*. Presumably, these extracts contain several enzymic activities (Dicker, 1960) directed towards either the disulphide or amide bonds contained in the vasopressin molecule. Further, it has been suggested that these enzymes are responsible for the inactivation of vasopressin *in vivo*. In view of the demonstration that many tissues contain a spectrum of hydrolytic enzymes whose activities become apparent only after disruption of the cell or particles, or both, contained within the cell (de Duve, 1959), there is a reasonable possibility that such enzymes present in tissue extracts may either have no relationship to or mask similar enzymic activities operative in the intact cell. Therefore, in order to study the manner in which the mammalian kidney inactivates vasopressin, it was decided to use rat-kidney slices in

these investigations. Such preparations *in vitro* have proved to be of value in studies concerned with renal tubular transport (Taggart, 1958). In this paper are the first phases of these studies where we have attempted to define some of the properties and requirements for the inactivation of arginine vasopressin *in vitro*, by rat-kidney slices.

It was expected (and subsequently demonstrated) that the incubation of kidney slices could under some circumstances give rise to the presence of enzymes in the incubation medium capable of inactivating vasopressin. Since such a situation would be analogous to the studies conducted by Dicker (1960) with cell-free preparations, it was essential to find conditions where the intact slice was solely and intimately associated with the disappearance of vasopressin activity from the incubation medium. Under our standard incubation conditions, which included the use of slices previously incubated in buffer alone, and an incubation temperature of 25°, it could be shown that this requirement was essentially fulfilled.

Aerobic phosphorylation, an important requirement for active transport by the kidney cell, was apparently not a rate-limiting step in the inactivation of vasopressin. Some of the observations on the rate of vasopressin inactivation by kidney slices may be summarized: the overall velocity followed first-order kinetics; the apparent first-order rate constant was proportional to kidney-slice concentration and decreased with increasing substrate concentration; the vasopressin concentration which gave half-maximal velocity was approximately 55 μM; and a 10° rise in temperature (over the range studied) gave an approximate doubling of the reaction rate. These results are reminiscent of the properties of a number of well-characterized proteolytic enzyme systems (cf. Elkins-Kaufman & Neurath, 1948; Neurath & Schwert, 1950; Adams & Smith, 1951). However, supplementing the incubation medium or pre-incubating slices with a number of reagents (previously shown to inhibit the action of a variety of such proteases or enzymes, or both, capable of inactivating vasopressin) failed to affect the rate of disappearance of vasopressin from the incubation medium. Nevertheless, the inability of these inhibitors to affect the rate of inactivation of vasopressin in a crude system does not preclude the possibility that such enzymes may play an important role in the inactivation of vasopressin by the kidney cell. In any event, valid conclusions concerning the kind of enzymic activities involved would require an identification of the reaction products.

The possibility remained that the vasopressin which disappeared from the incubation medium was taken up and stored by the kidney slice in a manner analogous to that observed *in vivo* with adrenocorticotrophic hormone (Richards & Sayers, 1951) and other foreign proteins (Oliver, MacDowell, Moses & Lee, 1954), although vasopressin storage seemed unlikely (Heller & Zaidi, 1957). Trichloroacetic acid extracts of slices previously incubated with vasopressin did not contain significant quantities of pressor activity. The extraction procedure used was previously shown to be effective in the recovery of vasopressin from brain tissue (Sachs, 1960*a*), from blood (Weinstein, Berne & Sachs, 1960) and from kidney slices homogenized in the presence of vasopressin and trichloroacetic acid.

Also, the biological recovery experiments were confirmed by the use of [^{35}S]vasopressin prepared biosynthetically. Speculations on the precise nature of the inactivation products would at this point be premature.

SUMMARY

1. Highly purified arginine vasopressin, in the presence of rat-kidney slices, was shown to disappear at a rapid rate from the incubation medium.

2. The disappearance of vasopressin from the incubation medium was not due to the presence of degradative enzymes in the medium which were liberated from either broken or intact cells.

3. The apparent first-order rate constant was consistently reproducible with slices taken from different animals on successive days and was shown to be proportional to the weight of kidney tissue.

4. Above 3 i.u. of vasopressin/ml., the apparent k_{75} fell progressively as the concentration of hormone was increased; half-maximal velocity was achieved at a concentration of 55 μM.

5. Below pH 6·0, little or no loss of vasopressin occurred, whereas the optimum rate of disappearance of hormone was observed over the pH range 7–10.

6. The mean k_{75} values were found to be 0·0109 $\pm$ 0·0005 min.$^{-1}$ at 25° and 0·0026 $\pm$ 0·004 min.$^{-1}$ at 7°.

7. Dinitrophenol (0·1 or 1 mM), di*iso*propyl phosphorofluoridate (1 mM), trypsin inhibitor (1 mg./ml.), ethylenediaminetetra-acetate (0·04M), and phosphate (0·2M) did not affect the rate of loss of vasopressin from the incubation medium; *p*-chloromercuribenzoate (1 mM) and oxytocin (15 i.u./ml.) induced 30 and 50% inhibition respectively.

8. The vasopressin which disappeared from the incubation medium could not be recovered from the kidney slice and had apparently undergone inactivation. This was confirmed by the use of [^{35}S]vasopressin prepared biosynthetically.

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Acidic Peptides of the Lens

7. THE PREPARATIVE ENZYMIC SYNTHESIS OF OPHTHALMIC ACID*

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Ophthalmic acid (γ-glutamyl-α-amino-*n*-butyrylglycine) is a peptide isolated from calf lens (Waley, 1958). This analogue of glutathione has been used in studies on *Hydra* (Cliffe & Waley, 1958*a*), on the metabolism of γ-glutamyl peptides (Cliffe & Waley, 1961*a*), on the glyoxalase reaction (Cliffe & Waley, 1959, 1961*b*) and on embryological development (Lallier, 1959). The need for a method of producing ophthalmic acid is not altogether met by the synthesis reported by Waley (1958), which is fairly lengthy and requires the expensive L-α-amino-*n*-butyric acid. The enzymic synthesis of ophthalmic acid from the constituent amino acids (Cliffe & Waley, 1958*b*; 1961*a*), in which DL-α-amino-*n*-butyric acid is used, has now been adapted to the production of this tripeptide.

The source of enzyme for the synthesis is rabbit liver, supplemented by lens (Cliffe & Waley, 1961*a*). The main problem is the isolation of the ophthalmic acid. This has been accomplished by ion-exchange chromatography. The method was based on the carrier-displacement technique (Buchanan, 1957). The separation of aspartic acid and glutamic acid was effected by Buchanan by adsorption on an anion-exchange resin in the acetate form; this column was then fitted over another column that contained the resin in the lactate form. When both columns were eluted with aqueous citric acid, the glutamic acid and aspartic acid emerged in that order, separated by a band of lactic acid ('the carrier'). When the same method was tested on a mixture of glutamic acid and ophthalmic acid, separation was effected, although lactic acid did not intervene between the amino acid and the peptide. Evidently ophthalmic acid is not acidic enough to be strongly retained by the lactate column. This trial experiment was carried out with about 5 mg. of ophthalmic acid and 50 mg. of glutamic acid on a column of 0·25 cm.^{2} cross-sectional area. The fractions that contained the ophthalmic acid were extracted with amyl alcohol, and with ethyl

* Part 6: Cliffe & Waley (1961*a*).

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acetate, to remove as much as possible of the lactic acid and citric acid; the peptide was then precipitated from solution with acetone. This material was free from glutamic acid, so that the resolution is adequate even with high loading of the column. The method was applied to the isolation of ophthalmic acid from the enzymic reaction mixture. The solution (after removal of protein, and of trichloroacetic acid) was freed from inorganic salts (and organic phosphates) by adsorption on a cation-exchange resin, which was washed with water and eluted with aqueous pyridine. Tests showed that there was no hydrolysis of ophthalmic acid during this procedure, which was carried out expeditiously. The method used for desalting was also useful for the final separation of ophthalmic acid (after the ion-exchange chromatography) from the lactic acid and citric acid in the eluate: the peptide crystallized readily from a concentrated aqueous solution on dilution with acetone.

The identity of the specimens of ophthalmic acid (*a*) isolated from calf lens, (*b*) synthesized non-enzymically and (*c*) synthesized enzymically, has already been shown by X-ray crystallography and has now been confirmed by infrared spectroscopy. The enzymic synthesis evidently utilizes only the L-isomer of DL-α-aminobutyric acid, since the product has the same specific rotation as the specimen synthesized (non-enzymically) from L-α-aminobutyric acid. We had earlier inferred that this was so, because the rate of synthesis of ophthalmic acid was the same whether DL-α-aminobutyric acid was used at one concentration or the L-isomer at half the concentration (Cliffe & Waley, 1958*b*).

The overall yield of ophthalmic acid in the enzymic synthesis was about 20% (based on L-α-aminobutyric acid): this yield could probably be improved if purified enzymes were used and, in particular, if γ-glutamyl lactamase (which diverts some of the intermediate, γ-glutamyl-α-aminobutyric acid, to 5-oxopyrrolidine-2-carboxylic acid) could be removed from the enzyme preparation.

MATERIALS

Enzyme preparation from liver. Rabbit liver was macerated under acetone at −10° (10 ml./g. of liver), collected at the pump, washed with cooled acetone and dried. The enzyme preparation was made from the powder by the procedure of Snoke & Bloch (1955); 24 g. of powder gave 210 ml. of enzyme preparation. The preparation may be stored at −15° for 3 weeks.

Dialysed lens extract. Calf lenses (20 g.) (stored at −15° for up to 4 weeks) were ground with cooled water (20 ml.) and a little sand. The mixture was dialysed for 48 hr. at 4° and then centrifuged at 3000*g* for 10 min. The supernatant may be stored frozen for up to 1 week without appreciable loss of activity.

Potassium salt of 3-phosphoglyceric acid. The barium salt (L. Light and Co. Ltd, Colnbrook, Bucks.) (14 g.) was triturated with water and Amberlite IR-120 (H^+) (200 ml.) was added. Portions of 5N-KOH were added until the pH rose to 6·4; after each addition of alkali, 3 min. was allowed for equilibration (Wade & Morgan, 1955). The mixture was transferred to a column, the solution run off and the resin washed twice with water (100 ml.). The combined solution and washings were concentrated under reduced pressure to 35 ml.

Solution of amino acids. A solution of glycine (2·25 g.), L-glutamic acid (2·942 g.) and DL-α-aminobutyric acid (4·124 g.) in water was neutralized with N-KOH (20 ml.) and made up to 100 ml.

METHODS

Incubation mixture. The components were: sodium salt of adenosine triphosphate (Sigma Chemical Co.) (1 g.) in water (40 ml.); 3-phosphoglyceric acid (potassium salt) soln. (12 ml.); M-KCl (36 ml.); M-$MgSO_4$ (4 ml.); amino acid soln. (20 ml.); M-KCN (6 ml.); 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (44 ml.) [the buffer contained tris (12·1 g.) and N-HCl (60 ml.) in 100 ml.]; dialysed lens extract (32 ml.); enzyme preparation from liver (200 ml.); water (6 ml.). The mixture (total volume 400 ml.) was incubated at 37° for 6 hr. at pH 7·8. Potassium glutamate [from L-glutamic acid (0·588 g.) and N-KOH (4 ml.) in 10 ml.] was then added, and the incubation continued for a further 3 hr.

Removal of protein. At the end of the incubation, cooled 15% (w/v) trichloroacetic acid (400 ml.) was added; the mixture was kept at 4° for 1 hr. and then centrifuged at 3000*g* for 10 min. The precipitate was washed with 7·5% (w/v) trichloroacetic acid (400 ml.). The combined supernatant solutions were extracted with *N*-methyl dioctylamine (British Hydrological Corp., Merton, Surrey) in $CHCl_3$ (10%, w/v; 1·5 l.) to remove trichloroacetic acid, and then with $CHCl_3$ to remove excess of base. The volume of the aqueous layer was then decreased to 300 ml. by distillation under reduced pressure.

Removal of salt. The aqueous solution was made to flow through a column 4·1 cm. × 10 cm. long of Dowex 50 (H^+) (4% cross-linked; minus 400 mesh; Bio-Rad Laboratories, Berkeley, Calif., U.S.A.; analytical grade). The column was then washed with water (about 150 ml.) until the pH of the effluent rose to about 3. The application of the sample to the column, and the washing, could be hastened by applying pressure to the top of the column. The amino compounds were eluted with 2M-pyridine; they started to come off just before the yellow band on the column, and continued rather after it. The ninhydrin-positive fractions were collected and concentrated; water was added, and removed by distillation several times, to remove pyridine. After drying *in vacuo*, a solid residue (2·5 g.) was obtained.

Anion-exchange chromatography. The anion-exchange resin, Dowex 1 (4% cross-linked; 200–400 mesh; Bio-Rad Laboratories; analytical grade) was converted from the chloride into the lactate form as follows. The resin was washed with water by decantation until the supernatant was clear, and then transferred to a Büchner funnel and washed with 2M-sodium lactate until the effluent no longer contained appreciable amounts of Cl^- ion, and finally was washed thoroughly with water. The acetate form of the resin was prepared by an analogous method.

The columns were packed with aqueous suspensions of the resins.

The desalted sample (2·5 g.) in water (100 ml.) was treated with pyridine until green to bromocresol green, and added to a column 1·6 cm. × 21 cm. long of Dowex 1 acetate during ¾ hr. The column was then washed with water (150 ml.); the effluents were rejected. This column, which contained the acidic amino compounds (glutamic acid and ophthalmic acid) was then fitted over a second column, 1·6 cm. × 65 cm. long, of Dowex 1 lactate. The tip of the bottom of the acetate column was slipped into a piece of plastic tubing fitted to the top of the lactate column, and filled with water so that there were no air bubbles between the columns. The columns were eluted overnight with citric acid (63 g.) in water (1500 ml.); the flow rate was 80 ml./hr. and fractions of 20 ml. were collected.

The ninhydrin-positive fractions were examined by paper electrophoresis at pH 4 (Cliffe & Waley, 1958*b*); tube 36 contained glutamic acid and tubes 37 and 38 contained ophthalmic acid. The effluent volume at which glutamic acid and ophthalmic acid are eluted varied in different experiments, but glutamic acid always emerged just before ophthalmic acid and there was never appreciable contamination.

Isolation of ophthalmic acid. Cation-exchange resin (Dowex 50) was washed with 2M-pyridine and then with water; it was converted into the acid form by washing with 3N-HCl and then with water. This resin was then made into a column 0·56 cm. × 12 cm. high. The fractions that contained ophthalmic acid were made to flow through the column (under pressure) at a rate of 1 ml./min. The column was washed with water during ¾ hr. and then eluted with 2M-pyridine. Fractions of 3 ml. were collected. The ninhydrin-positive fractions were evaporated to dryness *in vacuo*. The residue in water (0·8 ml.) was diluted to 3·5 ml. with acetone and the solution kept at room temperature for 48 hr. Ophthalmic acid (0·2 g.) separated; a further quantity (0·04 g.) was obtained from the mother liquors after dilution with more acetone (total yield 20%, based on DL-α-aminobutyric acid); $[\alpha]_D^{22}$ in 10% (v/v) acetic acid $-26 \pm 1°$ (*c*, 3). Under the same conditions, a synthetic specimen (Waley, 1958) had $[\alpha]_D^{22} - 27 \pm 1°$. The infrared spectrum of the specimen isolated from the enzymic synthesis was the same as the spectrum of the authentic specimen.

SUMMARY

1. The enzymic synthesis of ophthalmic acid from L-glutamic acid, DL-α-amino-*n*-butyric acid and glycine has been carried out on a preparative scale. Batches of about 400 ml. of reaction mixture yield about 200 mg. of ophthalmic acid.

2. The product has been isolated by ion-exchange chromatography and purified by recrystallization.

3. The enzymic synthesis is stereospecific and this enables racemic α-aminobutyric acid to be used.

We should like to thank our colleagues at this Laboratory for advice and encouragement, the staffs of the Oxford Co-operative and Eastwyck Farm Slaughterhouses for their co-operation and the National Council to Combat Blindness Inc., New York, for a Fight for Sight Grant for the purchase of chromatographic equipment. One of us (E. E. C.) was in receipt of a Thornton Bursary.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **402nd Meeting** *of the Society was held in the Courtauld Hall, Queen Elizabeth College, London, W. 8, on Saturday, 28 January 1961, when the following papers were presented:*

COMMUNICATIONS

(1) Effects of Protamine and Gangliosides on Ion Movements in Isolated Cerebral Tissues

By R. J. Woodman and H. McIlwain (*Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, London, S.E. 5*)

On applying electrical pulses to sections of guinea-pig cerebral cortex in oxygenated glucose–bicarbonate media their content of Na^+ increased, and their content of K^+ fell from 60 ± 4 (8) to 49 ± 4 (8) μequiv./g. wet wt. in the course of 10 min. (Cummins & McIlwain, 1960). Rapid re-entry of K^+ ensued when pulses were stopped, the average rate during the first minute being 600 μequiv./g./hr.; this re-entry did not occur if glucose was lacking, nor if 30 μM-2:4-dinitrophenol was present.

Re-entry of K^+ was inhibited by the relatively low concentrations of clupein sulphate (0·1 mg./ml.) which inhibited respiratory response to pulses (McIlwain, 1959). These concentrations had little effect on tissue K^+ in the absence of pulses, nor did they prevent the loss in K^+ caused by pulses. They however preserved a large part of the phosphocreatine in the tissue from the diminution which usually accompanied the application of pulses.

Protamine no longer inhibited re-entry of K^+ when gangliosides were added [0·3 mg./ml. of preparations containing 26–29% of *N*-acetylneuraminic acid; for other interactions with cerebral tissues see McIlwain (1960)]. This was not simply due to combination between protamine and gangliosides apart from the tissue, for protamine acted also when tissue was exposed to it for only 15 min.; tissue then rinsed free from protamine-containing medium and incubated in fresh media remained inhibited. If, however, this second medium contained gangliosides, normal movements of K^+ were observed.

It is proposed that the native gangliosides of normal tissue offer acidic sites which function in relation to the active cation transport which utilizes energy-rich phosphate. These sites are pictured to be occupied preferentially by the polybasic protamine.

Cummins, J. T. & McIlwain, H. (1960). *Biochem. J.* **76**, 64P; and in the Press.
McIlwain, H. (1959). *Biochem. J.* **73**, 514.
McIlwain, H. (1960). *Biochem. J.* **76**, 16P.

(2) Attachment of Protamine and Ganglioside to Cerebral Tissues and the Nature of their Effects on Ion Transport

By H. McIlwain and S. Balakrishnan (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, S.E. 5*)

During experiments similar to those of Woodman & McIlwain (1961), tissues incubated with protamine increased in arginine content to an extent equivalent to the attachment of about 6 mg. of protamine/g. of tissue. Incubation with gangliosides or suramin, though restoring excitability and ion movement, did not diminish the protamine of the tissue (Thomson & McIlwain, 1961).

The tissue content of ganglioside-like substances has now been determined by estimating *N*-acylneuraminic acid after solvent extraction and partition (Long & Staples, 1959; McIlwain, 1961).

Incubation with protamine, or keeping at 0°, both of which cause loss of tissue excitability (McIlwain, 1959), led also to a fall in the extractable ganglioside of the tissue. Incubation with added ganglioside had little effect on the extractable ganglioside of normal tissue, but increased that extractable from tissue incubated with protamine or kept at 0°.

Moreover suramin, which also restored excitability to tissue incubated with protamine, increased the ganglioside extractable from normal and from protamine-treated tissue. The Na^+- and K^+-activated adenosine triphosphatase of the tissue (Skou, 1957; Hess & Pope, 1957; J. Järnfelt, personal communication) has been found (D. H. Deul & H. McIlwain, unpublished results) to be inhibited by suramin and to be present in the membrane-derived tissue fractions rich in gangliosides (Wolfe & McIlwain, 1961; Wolfe, 1960). A mechanism of ion transport is proposed (McIlwain, 1960) in which a basic group of the active centre of the adenosine triphosphatase acts in association with a ganglioside-like membrane constituent, in extruding Na^+ to a hydrophilic path through the lipid of the cell membrane during the hydrolysis of adenosine triphosphate.

Hess, H. & Pope, A. (1957). *Fed. Proc.* **16**, 196.
Long, C. & Staples, D. A. (1959). *Biochem. J.* **73**, 385.

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McIlwain, H. (1961). *Biochem. J.* **78**, 24.
Skou, J. C. (1957). *Biochim. biophys. Acta*, **23**, 394.
Thomson, C. G. & McIlwain, H. (1961). *Biochem. J.* (in the Press).
Wolfe, L. S. (1960). *Biochem. J.* **77**, 9P.
Wolfe, L. S. & McIlwain, H. (1961). *Biochem. J.* **78**, 33.
Woodman, R. J. & McIlwain, H. (1961). *Biochem. J.* **79**, 1P.

(3) An Enzymic Assay for Pyridine Nucleotides in Extracts of Animal Tissues

By Claude A. Villee (*Department of Biological Chemistry, Harvard Medical School, Boston* 15, *Mass., U.S.A.*)

An enzymic method for the assay of oxidized and reduced di- and tri-phosphopyridine nucleotides (DPN, DPNH, TPN, TPNH) was described by Glock & McLean (1955). This has been modified, using diaphorase and dichlorophenolindophenol in place of cytochrome *c* reductase and cytochrome *c*, so that the assays can be carried out with commercially available enzyme preparations. Samples of tissue are homogenized in hot 0·1N-HCl or 0·1N-NaOH, chilled, neutralized and centrifuged at high speed. Portions of the extracts are incubated with an excess of TPN-specific glucose 6-phosphate dehydrogenase (Sigma Chemical Co.) or with DPN-specific alcohol dehydrogenase (Sigma), together with an excess of diaphorase (Nutritional Biochemicals Corporation) and 2:6-dichlorophenol-indophenol. The rate of decrease in extraction at 600 mμ is measured spectrophotometrically. The diaphorase, prepared from *Clostridium kluyveri*, will react with either DPNH or TPNH. In measuring the amount of reduced pyridine nucleotide present in a tissue extract by this system, both DPNH and TPNH are quickly oxidized by the excess of diaphorase and dye. However, no further reduction of the dye occurs unless the pyridine nucleotides are reduced by their specific dehydrogenases. In the presence of an excess of DPN-specific alcohol dehydrogenase and ethanol, the rate of subsequent reduction of the dye is a function of the amount of DPN present; in the presence of an excess of TPN-specific glucose 6-phosphate dehydrogenase and glucose 6-phosphate, the rate of subsequent reduction of the dye is a function of the amount of TPN present. Results are corrected for the small, separately determined rate of non-enzymic reduction of the dye by the tissue extracts alone and for the small, separately determined rate of dye reduction by a combination of diaphorase, dehydrogenase and substrate in the absence of the tissue extract. Analyses of rat liver, kidney, and muscle gave values which agreed with those obtained by Glock & McLean (1955).

Glock, G. E. & McLean, P. (1955). *Biochem. J.* **61**, 381.

(4) The Role of Glycolysis in the Acute Stimulation of Oxygen Consumption in the Whole Animal by Noradrenaline and Adrenaline

By H. Baum, R. E. Moore and H. A. El-Khanagry (*Departments of Biochemistry and Physiology, Royal Free Hospital School of Medicine, London, W.C.* 1)

In connexion with a theory of the mechanism of thermoregulation in the neonate (Moore & Underwood, 1960*a*) it has been shown that several species of newborn small mammals respond rapidly to a subcutaneous injection of noradrenaline by an increase of up to 200% in their oxygen consumption. A similar response is obtained with isoprenaline and dopamine but not with adrenaline, *O*-methylated noradenaline or *O*-methylated adrenaline (Moore & Underwood, 1960*b*). The effect disappears with age and although adult animals respond to adrenaline with a small and prolonged rise of oxygen consumption, this response is probably an indirect result of lactic acidaemia (Lundholm, 1949), and is not of the same magnitude as the noradrenaline response in the neonate.

We think that the difference in response of the neonate to noradrenaline and adrenaline, and the lack of response of the adult to either is a function of accessibility of the catecholamines to the site of action. An alternative possibility was that the mechanism of metabolic stimulation is insensitive to adrenaline and loses its sensitivity to noradrenaline with advancing age. This was ruled out by the observations that after pre-treatment with the adrenergic blockading agent dibenzyline, the newborn cat becomes responsive to adrenaline and the adult rat to both noradrenaline and adrenaline (Baum, Moore & Underwood, 1960).

It was possible that the metabolic stimulus was an indirect result of the products of stimulation of glycolysis. We have, therefore, measured oxygen consumptions and determined glucose and lactic acid in the blood of animals after catecholamine treatments. The results show that elevation of blood glucose and lactic acid can be obtained under conditions where there is no increase in oxygen consumption. Moreover, the blood glucose and lactic acid is not necessarily raised in animals with stimulated oxygen consumptions.

Stimulation of a neonatal cat by noradrenaline could not be mimicked by intravenous infusion of even large quantities of lactate, glucose or glucose plus insulin. We, therefore, conclude that this

catecholamine stimulation of metabolism is not an indirect result of the stimulation of glycolysis.

Baum, H., Moore, R. E. & Underwood, M. C. (1960). *J. Physiol.* **154**, 49*P*.
Lundholm, L. (1949). *Acta physiol. scand.* **19**, Suppl. 67.
Moore, R. E. & Underwood, M. C. (1960*a*). *Lancet*, **1**, 1277.
Moore, R. E. & Underwood, M. C. (1960*b*). *J. Physiol.* **153**, 67*P*.

(5) The Effect of Compounds Related to Vitamin B_{12} on the Metabolism of Propionate by *Ochromonas malhamensis*

By H. R. V. Arnstein and A. M. White (*National Institute for Medical Research, London, N.W.* 7)

Cell suspensions of *Ochromonas malhamensis* grown with limiting amounts of cyanocobalamin (0·03 μmg./ml.) oxidize sodium [2-^{14}C]propionate to CO_2 only at a slow rate (White & Arnstein, 1961). Addition of cyanocobalamin at optimum concentrations of 0·1–1 μg./ml. increases the rate more than 100-fold. After incubation, both methylmalonate and succinate were labelled, but in the absence of the vitamin succinate contained negligible radioactivity. Propionate metabolism thus presumably involves the pathway,

propionyl-CoA → methylmalonyl-CoA → succinyl-CoA,

which requires a coenzyme form of vitamin B_{12} for the last step (Stadtman, Overath, Eggerer & Lynen, 1960).

The specificity of the requirement for vitamin B_{12} has been investigated by measuring the oxidation of propionate by B_{12}-deficient cell suspensions (1–3 mg. of protein/ml.) incubated in water for 2 hr. at 30° after adding various compounds related to cyanocobalamin. At 1 μg./ml., factor A (2-methyladenylcobamide), factor B (cobinamide), pseudovitamin B_{12} (adenylcobamide) and the 6-hydroxy-2-methylmercaptopurine analogue were inactive, but the benzimidazole, dichlorobenzimidazole and hydroxybenzimidazole (vitamin B_{12III}) analogues had 51, 18 and 4% respectively of the activity of cyanocobalamin.

The methylamide and anilide analogues of cyanocobalamin, which inhibit the growth of this organism (Ford, 1959), were also tested. The anilide (10 μg./ml.) antagonized the stimulation of propionate oxidation by either cyanocobalamin or its coenzyme form (0·01 μg./ml.), but the methylamide had a marked vitamin B_{12}-like activity. Thus, 0·1 μg./ml. increased propionate oxidation from 0·5 to 35 μm-moles/hr./mg. of protein, corresponding to approximately 40% of that with cyanocobalamin. In other experiments, the methylamide had about 20% of the activity of vitamin B_{12}, and even at 100 μg./ml. propionate oxidation was stimulated. Similar effects were obtained with cells suspended in growth medium and under conditions where growth was shown to be inhibited.

Since the methylamide inhibits growth but stimulates oxidation, whereas vitamin B_{12III} stimulates the former and has only a small effect on the latter, it is concluded that vitamin B_{12} controls growth by a reaction other than the isomerization of methylmalonyl-CoA to succinyl-CoA.

Ford, J. E. (1959). *J. gen. Microbiol.* **21**, 693.
Stadtman, E. R., Overath, P., Eggerer, H. & Lynen, F. (1960). *Biochem. biophys. Res. Comm.* **2**, 1.
White, A. M. & Arnstein, H. R. V. (1961). *Biochem. J.* **78**, 10P.

(6) The Structure of Dermocybin

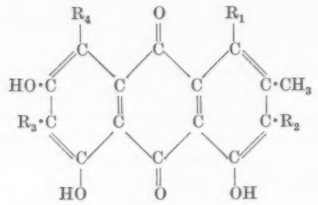
By J. H. Birkinshaw and Rachel H. Gourlay (*Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London, London, W.C.* 1)

Two crystalline anthraquinone derivatives, frangula-emodin (4:5:7-trihydroxy-2-methylanthraquinone), I, and dermocybin were isolated by Kögl & Postowsky (1925) from the dried sporophores of the fungus, *Cortinarius* (*Dermocybe*) *sanguineus* Wulf.

Dermocybin, $C_{16}H_{12}O_7$, is a red compound, having m.p. 228–229°, giving 2-methylanthracene on zinc-dust distillation and containing one methoxyl group and four acetylatable hydroxyl groups. Demethylation gives nordermocybin, $C_{15}H_{10}O_7$, m.p. 289°. These facts led Kögl & Postowsky (1925) to formulate dermocybin as a tetrahydroxymethoxy-β-methylanthraquinone.

Small quantities of dermocybin were isolated over a period of years in this department, but owing to the rarity of the fungus, accumulation of sufficient pigment was too slow for degradative studies and it was decided to approach the problem of structure mainly by synthetic methods. Spectroscopic evidence (Birkinshaw, 1955) is indicative of the presence of three free α-hydroxyl groups. Under conditions when only β-hydroxyl groups would be expected to react, dermocybin forms a monomethyl ether and a monoacetate and nordermocybin forms a dimethyl ether and a diacetate. This suggests that dermocybin has one free β-hydroxyl group and 3 α-hydroxyl groups whereas nordermocybin has 2 β-hydroxyl and 3 α-hydroxyl groups. Assuming that dermocybin is an emodin derivative, insertion of one α-hydroxyl group and one β-hydroxyl group into the emodin molecule

gives four possible structures for nordermocybin, namely, II, III, IV and V.



I, $R_1 = R_2 = R_3 = R_4 = H$
II, $R_1 = R_2 = OH$, $R_3 = R_4 = H$
III, $R_1 = R_3 = OH$, $R_2 = R_4 = H$
IV, $R_2 = R_4 = OH$, $R_1 = R_3 = H$
V, $R_2 = R_4 = OH$, $R_1 = R_3 = H$

3:4:5-Trimethoxyphthalic anhydride was condensed with *m*-cresol. The resulting benzoylbenzoic acid was oxidized by potassium persulphate and ring closure and demethylation of the product with conc. H_2SO_4 gave III, m.p. 298–300°. The absorption spectrum was closely related to that of nordermocybin but was not identical. Of the remaining structures II, IV and V, structure V, in which a hydroxyl group of III is transferred from the 1 to the 8 position, would be expected to have an absorption spectrum similar to III. Condensation of 1:2-dihydroxy-3:4-dimethoxybenzene (Baker & Jukes, 1934) with 3-hydroxy-5-methylphthalic anhydride (Raistrick, Robinson & Todd, 1933) and subsequent ring closure and demethylation with conc. H_2SO_4 gave V, 4:5:6:7:8-pentahydroxy-2-methylanthraquinone which agreed in all its properties with nordermocybin.

Dermocybin may then be represented as 4:5:7:8-tetrahydroxy-6-methoxy-2-methylanthraquinone or 4:5:6:8-tetrahydroxy-7-methoxy-2-methylanthraquinone.

Baker, W. & Jukes, E. H. J. (1934). *J. chem. Soc.* p. 1681.
Birkinshaw, J. H. (1955). *Biochem. J.* **59**, 485.
Kögl, F. & Postowsky, J. J. (1925). *Liebigs Ann.* **444**, 1.
Raistrick, H., Robinson, R. & Todd, A. R. (1933). *J. chem. Soc.* p. 489.

(7) Composition of Lipid from Protoplast Membranes and Whole Cells of *Micrococcus lysodeikticus*

By M. G. Macfarlane (*Lister Institute of Preventive Medicine, London, S.W.* 1)

Gilby, Few & McQuillen (1958) found that the membrane of protoplasts from *Micrococcus lysodeikticus* (8·6% of the bacterial dry weight) consisted of about 28% of lipid, 50% of protein and 20% of a mannose-containing carbohydrate; 80% of the lipid was a saturated phospholipid similar to cardiolipin.

The present work was done on protoplast membranes and whole cells of *M. lysodeikticus* N.C.T.C. 2665, prepared by Dr P. Mitchell and received frozen. The methods were those used by Macfarlane & Gray (1958); R_F values refer to chromatography on silicic acid paper at 2° in di*iso*butyl ketone–acetic acid–water (40:20:3, by vol.). The lipid was extracted from the membranes with 19 vol. of acetone–methanol (2:1, v/v), followed by 10 vol. of chloroform–methanol (2:1, v/v), and from the washed packed bacterial cells with 19 vol. followed by 4 vol. of chloroform–methanol (2:1, v/v). The lipid was separated, by dialysis in light petroleum (b.p. 60–80°) in a rubber finger, into a neutral lipid and a phospholipid fraction.

The material recovered from the membranes weighed 481 mg. (equivalent to about 5·6 g. dry wt. of bacteria), made up of: neutral lipid, 43 mg.; phospholipid, 133 mg. (P, 3·3; N, nil; carbohydrate, 4·6; Mg, 0·7%); fat-free residue, 251 mg. (N, 12·5; P, 0·19; Fe, 0·05; carbohydrate, 11%); proteolipid, 22 mg.; water-soluble material, 32 mg. The total from the whole cells was 21·3 g., made up of: neutral lipid, 142 mg.; phospholipid, 644 mg. (P, 3·0; N. 0·03; carbohydrate, 13·6; Mg, 0·6%); residue, 20·5 g. The lipid from the whole cells was little more than that of the protoplast membranes alone (36·8 and 31·4 mg./g. dry wt. of bacteria). The main constituent of the neutral lipid from both extracts was a diglyceride, with branched-chain saturated fatty acids, 77% of which were C_{15} acids.

The membrane phospholipid gave three spots on paper (R_F values 0·68, 0·45, 0·12; cardiolipin, 0·75; phosphatidylinositol, 0·12); chromatography on silicic acid gave (I) 68% of the P as diphosphatidylglycerol, with some phosphatidic acid; (II) 24% as a complex (R_F 0·45) giving on acid hydrolysis glycerophosphate, glycerol, mannose and fatty acids in molar ratio 1:1:1:4; (III) 8% as a mixture (R_F values 0·35, 0·12) containing some of II and about 5% of phosphatidylinositol. The phospholipid from the whole cells gave on paper only one spot, R_F 0·45; the material, however, was resolved on silicic acid, with some loss of carbohydrate, into diphosphatidylglycerol, carbohydrate-containing and inositol phosphatide fractions similar to those from the membranes. The phospholipid fatty acids were mainly (80–90%) branched-chain C_{15} acids, predominantly the *anteiso*-acid, 12-methyltetradecanoic acid. This is probably the main acid in *Sarcina* lipid (Akashi & Saito, 1960), the *iso*-acid, 13-methyltetradecanoic acid, being predominant in *Bacillus subtilis* and *B. natto* lipids (Saito, 1960).

The presence of the phosphatidylglycerols, glycolipid and a diglyceride with similar fatty acids in the protoplast membrane is of interest in

view of the metabolic activity of the phosphatidylglycerols in other organisms (cf. Benson & Strickland, 1960).

I am indebted to Dr P. Mitchell, Department of Zoology, University of Edinburgh, for the material, and to Dr G. M. Gray for gas-chromatography data. The support of the British Empire Cancer Campaign is gratefully acknowledged.

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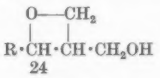
Benson, A. A. & Strickland, E. H. (1960). *Biochim. biophys. Acta*, **41**, 328.
Gilby, A. R., Few, A. V. & McQuillen, K. (1958). *Biochim. biophys. Acta*, **29**, 21.
Macfarlane, M. G. & Gray, G. M. (1958). *Biochem. J.* **70**, 409.
Saito, K. (1960). *J. Biochem., Tokyo*, **47**, 710.

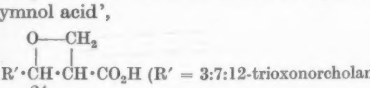
(8) The Chemical Nature of 'Scymnol Sulphate'

By T. Briggs* and G. A. D. Haslewood (*Guy's Hospital Medical School, London, S.E.* 1)

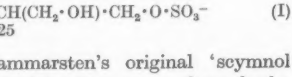
The alcohol sulphate of selachian bile on alkaline hydrolysis yields scymnol, of formula



(Cross, 1960) where R = 3α:7α:12α-trihydroxynorcholanyl. Cold chromic oxidation of scymnol gives 'scymnol acid',



in which the oxide ring is not opened (Bergmann & Pace, 1943). It was suggested (Haslewood, 1951) that the oxide ring in scymnol might be an artifact of alkaline treatment of the natural sulphate: in this case the native bile-salt ion would have the formula:



(I)

A sample of Hammarsten's original 'scymnol sulphuric acid Na salt', given to us through the kindness of Professor S. Bergström, gave a single spot on paper chromatography and failed to show the spectral band at 961 cm.$^{-1}$ assigned to the oxide ring in scymnol (Cross, 1960). Cold chromic oxidation of this sulphate gave dehydrocholic acid, R'·CO_2H, in fair yield. Since the oxide ring in 'scymnol acid' resists this treatment, it cannot be present in the sulphate, and we conclude that Hammarsten's sample is the sodium salt of (I).

We concur with a suggestion made to us by Dr A. D. Cross that scymnol should be renamed 'anhydroscymnol' and the native bile salt be called 'scymnol sulphate'. The new 'scymnol' is thus a hexahydric alcohol which remains to be isolated.

* This investigation was carried out during the tenure of a Postdoctoral Fellowship from the National Heart Institute, United States Public Health Service.

Bergmann, W. & Pace, W. T. (1943). *J. Amer. chem. Soc.* **65**, 477.
Cross, A. D. (1960). *Proc. chem. Soc.* p. 344.
Haslewood, G. A. D. (1951). *Symp. biochem. Soc.* **6**, 83.

(9) Biotin-sparing by Sorbitol

By T. B. Morgan and John Yudkin. (*Department of Nutrition, Queen Elizabeth College, University of London, London, W.* 8)

We have shown that animals fed a diet free from all vitamins of the B group can survive and grow for at least 12 months provided that the diet also contains sorbitol (Morgan & Yudkin, 1957). We have also shown that sorbitol specifically spares thiamine, riboflavin, pantothenic acid and pyridoxine (Hedley & Yudkin, 1959). Biotin, however, is not spared by sorbitol, according to the experiments of Herndon, Greenberg, Van Loon, Mathues & Parmalee (1959). Our own experiments do not confirm this.

Three groups of male albino rats aged six weeks were given diets containing avidin with all of the B vitamins except biotin. One group acted as control and received a supplement of biotin; a second group received 10% sorbitol; the third group received the biotin-free diet without sorbitol. After about 12 weeks, the animals in the third group showed definite signs of biotin deficiency. These signs were not seen either in the control rats receiving biotin, or in the rats receiving sorbitol and no biotin. After a further four weeks, the diets of some animals not receiving biotin were interchanged. Sorbitol was removed from the diet of two rats receiving it, and added to the diets of two rats not receiving it. Removal of sorbitol soon led to the development of signs of deficiency; the addition of sorbitol soon led to improvement in the signs of deficiency.

Hedley, V. M. & Yudkin, J. (1959). *Proc. Nutr. Soc.* **18**, xxiv.
Herndon, J. F., Greenberg, S. M., Van Loon, E. J., Mathues, J. K. & Parmalee, E. I. (1959). *Fed. Proc.* **18**, 529.
Morgan, T. B. & Yudkin, J. (1957). *Nature, Lond.*, **180**, 543.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **403rd Meeting** *of the Society was held in the Physics Lecture Theatre, Imperial College, London, S.W. 7, on Saturday, 25 February* 1961, *when the following papers were presented:*

COMMUNICATIONS

(1) Chromatography of ^{32}P-Labelled Deoxyribonucleic Acid, Synthesized in *Salmonella typhimurium* after Ultraviolet Irradiation

By T. Hudnik-Plevnik* and L. A. Stocken. (*Department of Biochemistry, University of Oxford*)

Kelner (1953) found that exposure of *Escherichia coli* B/r to moderate doses of ultraviolet light prevented their subsequent division without reduction in the cell mass. Kanazir & Errera (1956) confirmed this observation and Kanazir, Kacanski & Krajincanic (1957) obtained the same result with *Salmonella typhimurium* strain LT_2. These data suggested that deoxyribonucleic acid (DNA) synthesized after irradiation may be different from the normal one. Support for this idea was provided by Kanazir, Cecuk, Krajincanic & Hudnik (1959) who observed that whereas DNA from non-irradiated *S. typhimurium* could reduce the damage to an irradiated culture, that from an irradiated specimen could not.

Preliminary experiments by Hudnik and Glisin showed, by viscosity and ultraviolet-absorption measurements, that small changes in the heat-denaturation pattern can be detected in DNA synthesized after irradiation when compared with the DNA from control bacteria.

Ord & Stocken (1960) observed differences in the DNA obtained from thymus glands of control and irradiated rats, when chromatographed on ECTEOLA columns (Bendich, Pahl, Korngold, Rosenkranz & Fresco, 1958) and the same method was therefore used for characterization of DNA from normal *S. typhimurium* and of that synthesized in bacteria after they had been exposed to ultraviolet light.

In two experiments the ratios of the absorption at 260 mμ of the control to the irradiated DNA were: fraction 1*a*, 0·85, 0·80; fraction 1*b*, 0·98, 0·95; fraction 2, 1·10, 1·25; fraction 3, 0·98, 0·98; fraction 4, 1·08, 1·03. These data indicate that the relative amount of DNA from the irradiated bacteria is greater in fraction 1*a* and is at the expense of fraction 2. Since the bacteria had been grown in the presence of $^{32}PO_4^{3-}$ it was possible to measure the specific activities of the various fractions and it was of interest to note that differences were evident in this respect also.

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We are grateful to Dr J. Lascelles and Dr R. Tucker for help with the irradiation and growth of the organism. One of us (T.H.-P.) wishes to acknowledge financial support from the Institute of Nuclear Sciences, Belgrade, Yugoslavia.

Bendich, A., Pahl, H. B., Korngold, G. C., Rosenkranz, H. S. & Fresco, J. R. (1958). *J. Amer. chem. Soc.* **80**, 3949.

Kanazir, D., Cecuk, O., Krajincanic, B. & Hudnik, T. (1959). *Bull. Inst. Nuclear Sciences 'Boris Kidrich'*, **9**, 133.

Kanazir, D. & Errera, M. (1956). *Cold Spr. Harb. Sym. quant. Biol.* **21**, 19.

Kanazir, D., Kacanski, K. & Krajincanic, B. (1957). *Bull. Inst. Nuclear Sciences 'Boris Kidrich'*, **7**, 109.

Kelner, A. (1953). *J. Bact.* **65**, 252.

Ord, M. G. & Stocken, L. A. (1960). *Biochim. biophys. Acta*, **37**, 352.

(2) Oxaloacetic Acid in Enzymic Transamination

By Barbara E. C. Banks (*introduced by* Margaret Kerly). (*William Ramsay and Ralph Forster Laboratories, University College London, London, W.C.* 1)

Oxaloacetic acid may, in principle, exist in three forms, namely as keto and as *cis*- and *trans*-enols (hydroxymaleic and hydroxyfumaric acids). The literature (Michael & Bucher, 1896; Wohl & Oersterlin, 1901; Wohl & Claussner, 1907; Wohl & Lips, 1907) records the existence only of the two enolic acids in the solid state (m.p. 152° and 184°), but recent work (Gruber, Pfleiderer & Wieland, 1956) is at variance with this. The rapid, initial

drop in ultraviolet absorption on dissolving the solid in neutral buffer solution (0·1 M-phosphate, pH 7·4) is presumably due to conversion of the highly conjugated enol into the low-absorbing keto form. At equilibrium, the percentage enol has been estimated to be 14–17 % spectrophotometrically and by bromine titration. The rate of ketonization has been determined.

By analogy with α-oxoglutaric acid, which is overwhelmingly ketonic in solution, it would be predicted that the keto form of oxaloacetic acid would be the active species in enzymic transamination in the glutamic–aspartic system. Since tautomerization of oxaloacetic acid is by no means instantaneous, the keto–enol interconversion should become rate limiting at sufficiently high enzyme concentrations. The spectrophotometric enzyme assay procedure depends on the high ultraviolet absorption of oxaloacetic acid, relative to the other three substrates, and this, in turn, depends on the presence of the enol in solution. The rate of change of optical density (280 mμ) on adding a very high concentration of electrophoretically homogeneous glutamic–aspartic transaminase (from pig-heart muscle) to a mixture of aspartate and α-oxoglutarate, has been determined and compared with a theoretical plot, calculated on the assumption that the keto acid is liberated from the enzyme and subsequently undergoes enolization at a predetermined rate. The initial rate of change of optical density was found to be linear whereas the theoretical curve shows a marked initial lag. Reaction, therefore, appears to proceed via the intermediate enolate ion and not through the keto acid.

This result is in conflict with the commonly accepted mechanistic theories which involve hydrolysis of Schiff's base intermediates. In addition, the concentration of the enolate ion, at neutral pH values, is so low that it is hard to reconcile the observed initial rates of the reverse reaction (i.e. between glutamate and oxaloacetate) with the probability of encounter between the enzyme and the enolate ion.

Grateful acknowledgement is made of a Nuffield Foundation Research grant.

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Wohl, A. & Lips, C. H. (1907). *Ber. dtsch. chem. Ges.* **40**, 2294.

(3) A Possible Route for Malate Oxidation by *Chromatium*

By R. C. Fuller* and H. L. Kornberg.† (*M.R.C. Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford*)

Chromatium strain D was grown anaerobically in the light on malate or acetate as carbon sources. With the latter but not the former substrate, *iso*-citratase was adaptively formed, indicating the operation of the glyoxylate cycle during growth on acetate. Although growth on either substrate would necessitate intracellular oxidation of malate, extracts of the organisms were devoid of diphosphopyridine nucleotide (DPN)-linked malate dehydrogenase (Sisler & Fuller, 1959). Crude sonic extracts of *Chromatium* oxidized L-malate with uptake of 0·5 mol. of O_2/mol. of malate; the product formed was pyruvate. This oxidation, which was stimulated by phenazine methosulphate, was abolished by treatment with activated charcoal and restored by catalytic quantities of triphosphopyridine nucleotide (TPN); it was shown to be catalysed by 'malic enzyme' (Ochoa, Mehler & Kornberg, 1948) (1):

$$\text{L-malate} + \text{TPN}^+ \rightarrow \text{pyruvate} + \text{TPNH} + \text{H}^+ + \text{CO}_2 \quad (1)$$

Particulate malate oxidation systems, as described for *Micrococcus lysodeikticus* (Cohn, 1958) or *Pseudomonas ovalis* (Kornberg & Phizackerley, 1961) were not detected.

Although no labelled oxaloacetate was formed from labelled malate when carbon dioxide was trapped, both labelled oxaloacetate and aspartate were formed when carbon dioxide was not removed. By use of [^{14}C]bicarbonate, the formation of these labelled C_4-acids from unlabelled malate was shown to be due to carboxylation of pyruvate. The small amounts of [^{14}C]aspartate formed in the absence of added substrates were increased three- to four-fold by addition of either malate plus cofactors, or by pyruvate or phosphoenolpyruvate, and ten-fold when this latter substrate was supplemented with inosine diphosphate. This indicated that one route for the carboxylation of C_3-compounds to C_4-acids was the oxaloacetic carboxylase system of Utter & Kurahashi (1954). A 27-fold stimulation of [^{14}C]aspartate formation from [^{14}C]bicarbonate occurred in the presence of pyruvate and adenosine triphosphate (ATP): this was increased to 41-fold by addition of catalytic

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† Present address: Department of Biochemistry, University of Leicester.

quantities of acetyl-coenzyme A, and indicated that the pyruvate carboxylation reaction (2) of Utter & Keech (1960) plays a major role in the carboxylation of pyruvate in *Chromatium*:

$$\text{pyruvate} + CO_2 + \text{ATP} \xrightarrow{\text{acetyl-coenzyme A}} \text{oxaloacetate} + \text{ADP} + \text{P} \quad (2)$$

These findings suggest that, in the obligatorily anaerobic bacterium *Chromatium*, malate can be oxidized to oxaloacetate by a route in which carbon dioxide plays a catalytic role. The overall reaction, being the sum of reactions (1) and (2), is (3):

$$\text{L-malate} + TPN^+ + \text{ATP} \rightarrow \text{oxaloacetate} + \text{TPNH} + H^+ + \text{ADP} + \text{P} \quad (3)$$

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Utter, M. F. & Keech, D. B. (1960). *J. biol. Chem.* **235**, PC 17.
Utter, M. F. & Kurahashi, K. (1954). *J. biol. Chem.* **207**, 787, 821.

(4) Properties of a Glycerate-forming Enzyme from *Pseudomonas ovalis* Chester

By A. M. Gotto* and H. L. Kornberg.† (*M.R.C. Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford*)

Pseudomonas spp. growing on glycollate as sole carbon source adaptively form enzymes which catalyse the overall conversion of glyoxylate into glycerate (Kornberg & Gotto, 1959, 1961) (1):

$$2\text{CHO}\cdot CO_2H + \text{DPNH}‡ \text{ (or TPNH}‡) + H^+ \rightarrow CH_2(OH)\cdot CH(OH)\cdot CO_2H + DPN^+ \text{ (or } TPN^+) + CO_2 \quad (1)$$

The two enzymes responsible are (2) glyoxylate carboligase (Krakow & Barkulis, 1956; Krakow Hayashi & Barkulis, 1959), which catalyses the decarboxylative condensation of glyoxylate to tartronic semialdehyde:

$$2\text{CHO}\cdot CO_2H \xrightarrow[\text{thiamine pyrophosphate}]{Mg^{2+}} \text{CHO}\cdot CH(OH)\cdot CO_2H + CO_2 \quad (2)$$

and a second enzyme (3), which catalyses the reduction of tartronic semialdehyde to glyceric acid:

$$\text{CHO}\cdot CH(OH)\cdot CO_2H + \text{DPNH (or TPNH)} + H^+ \rightarrow CH_2(OH)\cdot CH(OH)\cdot CO_2H + DPN^+ \text{ (or } TPN^+) \quad (3)$$

These enzymes were separated from sonic extracts of glycollate-grown *Ps. ovalis* Chester by fractionation with protamine sulphate and ammonium sulphate, treatment with alumina Cγ-gel, and chromatography on diethylaminoethylcellulose. Glycerate formation from glyoxylate occurred only when both enzymes were combined and not when either was omitted. The enzyme catalysing reaction (3), purified 40-fold from the crude sonic extracts, rapidly oxidized DPNH or TPNH with concomitant reduction of tartronic semialdehyde, and did not catalyse the oxidation of DPNH or TPNH in the presence of oxaloacetate, glyoxylate, pyruvate, D-glyceraldehyde, glycolaldehyde, formaldehyde and glyoxal. Hydroxypyruvate was reduced at less than 3% of the rate observed with equimolar concentrations of tartronic semialdehyde. The initial rate of enzymic oxidation of reduced pyridine nucleotide was proportional to the concentration of enzyme, of tartronic semialdehyde and of DPNH or TPNH. Maximal rates were obtained at pH 8·7, the K_m for tartronic semialdehyde being 2×10^{-4} M and the K_m for DPNH $3{\cdot}5 \times 10^{-5}$ M. Tartronic semialdehyde reduction was partially inhibited in the presence of glycollate, fluoroacetate and glyoxylate. The enzyme also catalysed the oxidation of glycerate, measured as the reduction of 2:6-dichlorophenolindophenol, in the presence of catalytic amounts of DPN^+ or TPN^+: no reaction occurred when enzyme, or glycerate, or pyridine nucleotide, was omitted. The K_m for glycerate in this oxidation was 5×10^{-4} M. Since the well-established 'glyceric dehydrogenase' of plants (Stafford, Magaldi & Vennesland, 1954; Holzer & Holldorf, 1957) forms glycerate from hydroxypyruvate but does not react with tartronic semialdehyde, we suggest that this new microbial enzyme be named 'tartronic semialdehyde reductase'.

Holzer, H. & Holldorf, A. (1957). *Biochem. Z.* **329**, 292.
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Stafford, H. A., Magaldi, A. & Vennesland, B. (1954). *J. biol. Chem.* **207**, 621.

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‡ Reduced di- and tri-phosphopyridine nucleotides.

(5) Malate Oxidation by *Pseudomonas* spp.

By H. L. KORNBERG* and P. J. R. PHIZACKERLEY.† (*M.R.C. Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford*)

Although cell-free extracts of many pseudomonads, such as *Pseudomonas* KB1 (Kogut & Podoski, 1953; Kornberg & Madsen, 1958) and *Pseudomonas* B_2aba (Kornberg & Gotto, 1961) have been shown to be rich in the soluble diphosphopyridine nucleotide (DPN)-linked malate dehydrogenase, sonic extracts of *Ps. ovalis* Chester did not catalyse the reduction of oxaloacetate by DPNH or TPNH, but instead contained an enzyme system which oxidized L-malate with oxygen, ferricyanide, cytochrome *c* or 2:6-dichlorophenolindophenol as electron acceptors. This oxidizing system was confined to the pink particulate sediment obtained by centrifugation at 144 000*g* for 90 min.; the supernatant solution contained virtually all the TPN-linked malic enzyme (Ochoa, Mehler & Kornberg, 1948). The particulate sediment, washed with and suspended in 20 mM-phosphate, pH 7·5, catalysed the rapid oxidation of L-malate, with stoicheiometric uptake of 0·5 mol. of O_2/mol. of substrate; oxaloacetate was identified as the major product. The initial rate of this oxidation by O_2, which was not stimulated by addition of DPN^+ or TPN^+, was reduced by more than 80% when mM-KCN or 2 mM-amytal (cf. Ernster, Jalling, Löw & Lindberg, 1955) were added. The initial rate of reduction of 2:6-dichlorophenolindophenol by L-malate was most rapid at pH 7·2 and was proportional to the concentrations of added particles. The K_m for L-malate in this assay was 1×10^{-4} M. The reaction with 2:6-dichlorophenolindophenol was unaffected by 100 mM-KCN but was inhibited (50–60%) by 2 mM-amytal. Extracts of those pseudomonads rich in the soluble malate dehydrogenase (which was not inhibited by amytal) did not contain this particulate malate oxidation system. These results suggest that, in *Ps. ovalis* Chester, the oxidation of malate proceeds via a system closely bound to structural components of the cell, which differs from the particulate malate oxidation system of *Micrococcus lysodeikticus* (Gelman, Zhukova & Oparin, 1960) in being cyanide-sensitive and is thus possibly linked to cytochromes.

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Ernster, L., Jalling, O., Löw, H. & Lindberg, O. (1955). *Expt. Cell Res., Suppl.* **3**, 124.

Gelman, N. S., Zhukova, I. G. & Oparin, A. I. (1960). *Doklady Akad. Nauk, S.S.S.R.*, **133**, 1209.

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Ochoa, A., Mehler, A. H. & Kornberg, A. (1948). *J. biol. Chem.* **174**, 979.

(6) Isolation of the Cyclic Acetal of Glycerylphosphorylethanolamine from Ox-heart Lipids and its Probable Origin

By J. B. DAVENPORT and R. M. C. DAWSON. (*Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*)

In a recently developed technique for the determination of the individual tissue phospholipids by successive chemical hydrolyses (Dawson, 1960), a part of the fraction which was stable to mild acid and alkaline hydrolysis ('sphingomyelin' fraction) could not be identified with any known lipids. However, it was noticed that its concentration was directly proportional to the plasmalogen content of the tissue.

The alkali- and acid-stable phospholipid fraction of ox-heart muscle was purified by acetone precipitation and crystallization from pyridine to remove non-phospholipid contaminants, and the product separated by chromatography on a silicic acid column with a gradient of methanol in chloroform. This gave rise to four phosphorus-containing peaks (*A–D*), the final two (*C*, *D*) being incompletely separated. The major peak (*C*) was identified as sphingomyelin from its N/P ratio, the virtual absence of combined glycerol and the products formed on hydrolysis with methanolic HCl. The next largest peak (*B*) after crystallization from acetone contained 6·3% P and 3·3% N, N/P ratio 1·05. The substance gave a Schiff's reaction for aldehyde and on hydrolysis with mercuric chloride (Thannhauser, Boncoddo & Schmidt, 1951) gave a product which was chromatographically identified as glycerylphosphorylethanolamine. It could be distinguished from lyso-ethanolamine plasmalogen prepared from ox brain by the method of Rapport, Lerner, Alonzo & Franzl (1957) by its complete stability to acid hydrolysis at 37°. It gave an infrared spectrum virtually identical with that of synthetic 2-aminoethyl-*trans*-2:3-*O*-hexadecylidene glycerol 1-phosphate, kindly supplied by Dr T. H. Bevan.

Recently Pietruszko & Gray (1960) have described the formation of the cyclic acetal of glycerylphosphorylcholine on the alkaline hydrolysis of naturally occurring choline plasmalogen in which the aldehydogenic group is an $\alpha\beta$-un-

saturated ether. Under the conditions of alkaline hydrolysis used in the present work, it was not possible to demonstrate any conversion of lyso-ethanolamine plasmalogen into the cyclic derivative. Presumably, therefore, cyclic acetal formation on alkaline hydrolysis must occur at the moment of deacylation.

It has been observed, however, that the water-soluble phosphorus released on acid hydrolysis at 37° of the alkali-stable tissue phospholipids is increased by some 20% by the addition of $HgCl_2$ (5–15 mM) to the hydrolysis mixture even though prolonging the acid hydrolysis results in no further release of water-soluble phosphorus. This extra phosphorus liberated is in the same form (i.e. glycerylphosphoryl-choline, -ethanolamine) as that released from the plasmalogens by acid hydrolysis, and it results in a correspondingly large reduction in the unknown fraction in the alkali- and acid-stable phospholipids. Furthermore, cyclic acetal phospholipids are not decomposed by acid at this temperature either with or without mercuric ions. These results therefore strongly suggest that the cyclic acetal forms of plasmalogens can be formed during the acid hydrolysis of lysoplasmalogens, a reaction which can be prevented by the addition of Hg^{2+} to the hydrolysis medium.

We are indebted to Dr D. Chapman, Chemical Laboratories, University of Cambridge, for the determination of the infrared spectrum.

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Rapport, M. M., Lerner, B., Alonzo, N. & Franzl, R. E. (1957). *J. biol. Chem.* **225**, 859.
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(7) Oestrone Sulphate in Human Pregnancy Urine

By JEAN MCKENNA, E. MENINI and J. K. NORYMBERSKI. (*Medical Research Council's Chemical Pathology of Steroids Research Unit, Jessop Hospital for Women, Sheffield* 3)

According to circumstantial evidence man excretes oestrone predominantly as its glucosiduronic acid; excretion of oestrone as its hydrogen sulphate has been suggested (Katzman, Straw, Buehler & Doisy, 1954; Crepy, Jayle & Meslin, 1957; and references there cited). Direct evidence is now presented for the occurrence of oestrone hydrogen sulphate in the urine of pregnant women.

A concentrate of steroid hydrogen sulphates (McKenna & Norymberski, 1960) was prepared from the pooled urine of late human pregnancy. Treatment of the concentrate with acid yielded oestrone (8% of the total content of the urine) whilst treatment with β-glucuronidase was essentially ineffective. Further purification was brought about by repeatedly washing the concentrate with M-pyridinium sulphate, followed by countercurrent partition (24 transfers) in the system *tert.*-butanol–ethyl acetate–0·5% aqueous sodium chloride (30:70:100, by vol.) (Diczfalusy & Lauritzen, 1961), and finally by partition on a Celite column in the system toluene–butanol–3% aqueous ammonium hydroxide (70:30:100, by vol.) (Schneider & Lewbart, 1959). Two chromatographically discrete Kober chromogens were so isolated. One of them (the major component) was found identical with authentic oestrone hydrogen sulphate in the following respects: partition coefficients in two systems, R_F values in four systems, release by acid of oestrone as detected by paper chromatography and by Brown's method (Brown, 1955), positive tests on paper for sulphate with rhodizonic acid (Burma, 1953) and for phenol with Folin–Ciocalteu reagent (Folin & Ciocalteu, 1927) *after* solvolysis by dioxan–hydrochloric acid (Schneider & Lewbart, 1956).

After paper chromatography in the system di*iso*propyl ether–*tert.*-butanol–3% aqueous ammonium hydroxide (60:40:100, by vol.) (Schneider & Lewbart, 1959) the material exhibited an infrared spectrum identical in positions of main peaks with that of authentic ammonium oestrone sulphate.

The minor component was hydrolysed and the product chromatographed on paper in Bush's system B3 (Bush & Willoughby, 1957); its mobility was identical with that of oestradiol.

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(8) A Maltose–Glucose Transglucosylase from Wheat Germ

By J. EDELMAN and A. J. KEYS (*introduced by* HELEN K. PORTER). (*Department of Plant Physiology, and Agricultural Research Council Unit of Plant Physiology, Imperial College of Science and Technology, London, S.W.* 7)

Addition of uniformly labelled [^{14}C]glucose to phosphate buffer extracts of wheat germ, pH 7·2, caused rapid incorporation of ^{14}C to occur into a component present in the extract which was tentatively identified as maltose from its position on paper chromatograms. This incorporation was increased by addition of maltose to the reaction mixture and occurred without appreciable change in the concentration of either glucose or maltose. The activity was present in two samples of commercial wheat germ obtained from different sources, and also in extracts of embryos dissected from germinating grain. Active protein fractions were obtained by precipitation with ammonium sulphate, but no serious attempt was made to purify the enzyme.

Labelling of maltose was confirmed by isotope dilution: carrier maltose was added to the suspected radioactive maltose and maltosazone prepared by treatment with phenylhydrazine. The product was radioactive, and the level of its specific radioactivity did not decrease after repeated recrystallizations. Reduction of the radioactive maltose with sodium borohydride followed by acid hydrolysis of the resultant maltitol gave the expected mixture of glucose from the non-reducing moiety and sorbitol from the reducing moiety (Reynolds, Porter & Martin, 1958); only the sorbitol was labelled.

These results are interpreted as demonstrating enzymic transfer of the non-reducing glucosyl residue of maltose to free glucose to re-form maltose, this process occurring without appreciable hydrolysis of glucosidic linkages. This reaction affords a useful means of preparing maltose labelled in either of its glucose residues. D-Mannose, D-galactose, L-arabinose and D-xylose could not replace glucose as acceptor, nor could cellobiose, isomaltose, methyl α-D-glucoside or glucose 1-phosphate replace maltose as donor. A mixture of soluble starch and labelled glucose gave rise to labelled maltose, presumably because of prior hydrolysis of the starch by amylase to unlabelled maltose which then acted as donor.

Although this reaction is unusual among sugar transfers and its significance is as yet unknown, its mechanism closely resembles that of the 1^{F}-fructosylsucrose–sucrose transfructosylase described by Edelman & Recaldin (1961).

We thank Mr C. Heap for help with experimental work.

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(9) A 1^{F}-Fructosylsucrose–Sucrose Transfructosylase from Jerusalem Artichoke Tubers and its Involvement in Fructosan Synthesis

By J. EDELMAN and D. A. C. L. RECALDIN (*introduced by* HELEN K. PORTER). (*Department of Plant Physiology, Imperial College of Science and Technology, London, S.W.* 7)

Addition of [^{14}C]sucrose to tuber extracts containing native fructosan (Dedonder, 1950; Bacon & Edelman, 1951) caused ^{14}C to appear rapidly in the next higher member of the fructosan series, viz. 1^{F}-fructosylsucrose. The failure to observe concomitant production of [^{14}C]glucose eliminated the possible mediation of inulosucrase (Dedonder, 1952), or of invertase (Allen & Bacon, 1956; Pavlinova & Kursanov, 1956). There was no change in the concentrations of the reactants, and the only means of detecting the reaction has been by following the shift of ^{14}C from sucrose into the trisaccharide, or vice versa. In view of these observations, the transfer of terminal fructose residues from 1^{F}-fructosylsucrose to sucrose to reform the trisaccharide is postulated. This reaction is comparable to the one described in the previous communication (Edelman & Keys, 1961).

Ammonium sulphate precipitation gave highly active protein preparations. Using these it was shown that: the pH optimum was 6; there was no action on sucrose in the absence of the trisaccharide; action on 1^{F}-fructosylsucrose alone resulted in slight self-transfer to form traces of tetrasaccharide; a variety of sugars, sugar phosphates and nucleoside triphosphates did not replace sucrose as acceptor; 6^{G}-fructosylsucrose did not replace 1^{F}-fructosylsucrose as donor.

When uniformly labelled 1^{F}-[^{14}C]fructosylsucrose was added to tuber juice ^{14}C appeared rapidly in sucrose, and also in material which was tentatively identified as polysaccharide containing fructose. This material had zero R_F value on paper chromatograms; total hydrolysis by weak acid produced [^{14}C]fructose and a little unlabelled glucose, and partial hydrolysis gave radioactive products identified as oligofructosides by paper chromatography and radioautography. Such material of zero R_F value which contains fructose is henceforth referred to as polysaccharide. [^{14}C]-Polysaccharide was not formed when 1^{F}-fructosylsucrose labelled only in its sucrose residue was used

as substrate, showing that it is the terminal fructose residue of the trisaccharide which is involved in the polymerization.

Protein fractions obtained by ammonium sulphate precipitation were highly active in catalysing polysaccharide synthesis from 1^F-fructosylsucrose, and neither the homologous tetrasaccharide nor sucrose could replace the trisaccharide in this reaction. The possible requirement for a primer has not yet been ascertained. Polymer formation always ceased when about half of the transferable fructose had been utilized: this was ascribed to the by-production of sucrose, as addition of sucrose at the start of the reaction was strongly inhibitory. It is possible that 1^F-fructosylsucrose–sucrose transfructosylation and fructosan synthesis are expressions of the same enzymic activity.

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(10) Sucrose Synthesis in the Living Scutellum of Wheat Seedlings

By A. J. Keys and S. J. Skews (*introduced by* Helen K. Porter). (*Agricultural Research Council Unit of Plant Physiology, Imperial College of Science and Technology, London, S.W.* 7)

Radioactive uridine diphosphate glucose (UDPG) occurs in cereal seedlings after prolonged incubation with labelled glucose or fructose (Edelman, Shibko & Keys, 1959). When radioactive glucose or fructose was supplied to isolated wheat or barley scutella, sucrose was the main labelled product, sugar phosphates also becoming labelled (Edelman, *et al.*, 1959). Wheat scutella, therefore, seemed a suitable tissue to use for obtaining direct evidence about intermediate steps in the conversion of hexose into sucrose *in vivo*.

Scutella, sliced from 2-day germinated seedlings of 'Cappelle' wheat, were placed epithelium downwards in 0·01 ml. (approx. 30 μg. and 2 μc) of a solution of radioactive glucose or fructose at either room temperature (22°) or at between 2° and 5° for periods up to 20 min. Sugar not absorbed was washed away and the tissue killed in boiling ethanol. Labelled reaction products extracted by 50% aqueous ethanol were separated on Whatman no. 1 chromatography paper in one dimension by multiple development at 23°, using successively: ethanol–M-ammonium acetate buffer, containing 0·01 M-ethylenediaminetetra-acetic acid, at pH 3·8 (7·5:3, v/v) (Paladini & Leloir, 1952) for 6 hr., the same solvent for a further 6 hr., and finally butanol–acetic acid–water (4:1:5, by vol.) (Partridge, 1948) for 36 hr., drying between each development. Labelled reaction products were tentatively identified by reference to a control strip bearing known compounds.

In all cases glucose 6-phosphate, fructose 6-phosphate, UDPG and sucrose became labelled but no evidence was obtained of formation of sucrose phosphate. After 10 min. at 22° with glucose as substrate 3·7% of the label absorbed was in maltose (2% was in sucrose and 91% in glucose) but none in free fructose. The time sequence of appearance of activity in the several products suggests that formation of sugar phosphates precedes that of UDPG and sucrose.

The labelling of maltose when glucose is supplied is assumed to be a result of a transfer of a glucosyl group from native maltose to the labelled sugar. This reaction is discussed by Edelman & Keys (1961).

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(11) Purification of Bovine Thrombin by Adsorption on Glass

By J. Green. (*Department of Pathology, Paddington General Hospital, London, W.* 9)

Purification of commercial thrombin (Thrombin, Topical; Parke Davis and Co.) has been carried out by adsorption in a column of powdered glass, and elution with 0·75 M-NaCl. Glass powder (British Drug Houses Ltd.) is washed by decantation, cleaned with dichromate–sulphuric acid, then washed thoroughly with tap water, and rinsed in distilled water. The adsorptive capacity of a column of 100 g. of powdered glass is about 2000 National Institutes of Health units (Manufacturer's unitage), and in practice 100 mg. of thrombin is applied in 5 ml. of 0·85% NaCl, and washed through with cold distilled water. Adequate yields of thrombin are obtained by elution

with 0·75M-NaCl, provided the pH value of the effluent is kept above 6·5. Elution can be made almost quantitative by incorporating 50 mM-sodium dithiodiglycollate in the eluent but this may modify the properties of the eluted thrombin.

Separation of thrombin from eluent is carried out on a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden), and the thrombin precipitated by an equal volume of acetone at 0°, and freeze-dried. More than fivefold purification can be obtained. The purified material retains the tosyl-arginylmethyl esterase activity of the original. It enhances fibrinolytic activity in the system described previously (Green, 1959).

Electrophoresis on cellulose acetate shows two main bands, a diffuse one near the normal fibrinogen position, and a more mobile one, but clear separation of components has not been achieved.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **404th Meeting** *of the Society, the Fiftieth Anniversary and Annual General Meeting, was held in London on 27–29 March* 1961. *The Symposium was in the Beveridge Hall, Senate House, University of London, London, W.C.* 1, *on Monday and Tuesday, 27–28 March, and the Communications were presented in the Department of Biochemistry, University College London, Gower Street, London, W.C.* 1, *on Wednesday, 29 March.*

SYMPOSIUM ON 'THE STRUCTURE AND SYNTHESIS OF MACROMOLECULES'

The Structure of Polynucleotides and the Properties of Nucleic Acids

By PAUL DOTY. (*Department of Chemistry, Harvard University, Cambridge, Mass., U.S.A.*)

The enzymic synthesis of high-molecular-weight polyribonucleotides and polydeoxyribonucleotides has made possible the determination of the preferred structures of these simpler analogues of nucleic acid chains. This has led to greater insight into the structures of nucleic acids.

All homologous polynucleotides undergo self interaction leading to helical complexes in particular ranges of pH and ionic strength. Since all of these complexes are hypochromic in the ultraviolet their existence and breakdown are readily inferred from absorbance measurements. In this way the relative stability of each can be assessed and the kinetics of formation followed.

A number of two- and three-stranded helical complexes can be formed by mixing two different homopolymers under proper conditions. Stability studies show that two-stranded helices based on the Watson–Crick pairs are generally the most stable.

In copolynucleotides extensive base pairing occurs by the formation of short helical regions patterned after the deoxyribonucleic acid helix but it can be shown that a few bases can loop out of the helix in order to permit the Watson–Crick pairs to form in sequence. The stability of short helices is usefully studied with oligonucleotide fractions. From this it is clear that typical trinucleotides will form a stable complex with a complementary region of a polynucleotide or nucleic acid chain.

Against this background it is possible to show that ribonucleic acid contains these structures, but to a greater extent than expected from random sequence.

Further work has led to the formation of helices composed of one polyribonucleic acid chain and one polydeoxyribonucleic acid chain. Suggestive evidence for ribonucleic acid–deoxyribonucleic acid helical complexes *in vivo* is at hand.

Finally, these studies aid in understanding and exploiting the finding that single strands of deoxyribonucleic acid can be specifically re-formed into perfect helical complexes with a restoration of most of their biological activity.

Biochemical Aspects of Polynucleotide Biosynthesis

By J. N. DAVIDSON. (*Department of Biochemistry, University of Glasgow*)

The biosynthesis of polynucleotides takes place in three main stages: (*a*) the biosynthesis of nucleoside monophosphates, (*b*) their phosphorylation (usually to the triphosphate stage), (*c*) the polymerization of the nucleotide units into polynucleotides.

Polynucleotides are formed in four main types of reaction:

(i) Polynucleotide phosphorylase acts on ribonucleoside diphosphates to produce ribopolynucleotides with the elimination of inorganic phosphate. This enzyme occurs mainly in micro-organisms.

(ii) Enzymes present in a wide variety of living organisms can bring about the terminal addition to existing ribopolynucleotide chains of a limited number of polynucleotide units. The units to be added must be supplied as ribonucleoside triphosphates and inorganic pyrophosphate is liberated.

(iii) Several authors have described enzyme systems in ascites tumour cells, in liver, in calf-thymus nuclei, in chick embryo and in *Escherichia coli*, which can bring about the biosynthesis of ribopolynucleotides from the ribonucleoside triphosphates. The process can be illustrated either by net synthesis of ribopolynucleotide or by the non-terminal incorporation of labelled nucleotide units.

(iv) The Kornberg polymerase brings about the biosynthesis of deoxyribopolynucleotides from the

deoxyribonucleoside triphosphates with the elimination of inorganic pyrophosphate in presence of a suitable deoxyribopolynucleotide primer. Such an enzyme system has been investigated in cell-free extracts of *E. coli*, ascites tumour cells, thymus and regenerating rat liver. The mechanisms which control deoxyribopolynucleotide biosynthesis and the intracellular site at which it takes place were discussed.

The Structure of Polysaccharides

By E. L. HIRST. (*Department of Chemistry, University of Edinburgh*)

Knowledge of the molecular structure of the polysaccharides has been gained almost entirely during the life-time of The Biochemical Society. It is appropriate therefore to take this opportunity to review the growth of this knowledge and to consider some of the many problems still awaiting solution. As a result of the use of the original methylation method, supplemented by many physical, chemical and biological procedures, it is possible to obtain a general picture of the molecular structure of the polysaccharides, but in very few cases—perhaps none—can a unique detailed formula be assigned. To some extent this is inherent in the nature of the macromolecules. For example, in the case of cellulose, in addition to its polymolecularity, small variations—not necessarily the same in all molecules—may occur at irregular intervals along the chains of several thousand 1:4′-linked glucose residues. The idea that many polysaccharides may exist as families of closely related but not completely identical molecular species is still more evident in the hemicellulose group, where comparatively wide variations in attachment of short side chains to a linear framework of 1:4′-linked xylose residues are encountered. The precise location of these side chains along the main chain is unknown. Similar problems are met with in the structural chemistry of the starches and glycogens and, in still greater complexity, amongst the gums and mucilages. It is possible, however, that a greater degree of chemical homogeneity may be found in the *N*-containing polysaccharides and the immunopolysaccharides. But even if detailed molecular structures were known, much remains to be elucidated. For instance, it is becoming evident that polysaccharides are often laid down in Nature in structural patterns which markedly affect their properties—for example, the arrangement of the cellulose strands in the various layers of the plant cell wall. Furthermore, polysaccharides such as cellulose, pectic materials and hemicelluloses may well be linked chemically to other cell-wall components. Amongst other features of present-day interest, reference was made to the wide variety of sugar residues now known to occur in the polysaccharides, including sugars of both the D- and L-series, and numerous deoxy, oxidized, reduced and methylated derivatives of these sugars. Of special interest to the biologist are the mucopolysaccharides, the bacterial polysaccharides and the immunopolysaccharides, which are structurally extremely complicated and require new techniques for their investigation.

The Biosynthesis of Polysaccharides

By W. Z. HASSID. (*Department of Biochemistry, University of California, Berkeley, 4, U.S.A.*)

It has now been well established that complex saccharides are formed in living cells by transglycosylation reactions. In these reactions the carbohydrate moiety of a glycosyl donor is transferred to an acceptor, forming a new glycoside. The glycosyl donor may be a sugar phosphate such as α-D-glucose 1-phosphate, as in the formation of glycogen and starch by the phosphorolytic process; an oligosaccharide such as sucrose, from which the polysaccharides dextran and levan are formed; or a sugar nucleotide, primarily uridine diphosphate D-glucose, which is a substrate for the synthesis of a number of complex saccharides. Uridine diphosphate D-glucose is of particular importance, because the ΔF of hydrolysis of the glycosidic bond of the sugar nucleotide is greater than that of the other substrate donors. This causes the equilibrium of the reaction to favour the formation of the complex saccharide.

Sugar nucleotides containing D-glucose or other monosaccharides are present in animals, plants and micro-organisms. Most of them contain uridine as the nucleoside moiety. However, there are analogues in which guanosine, cytidine or thymidine is the nucleoside component. The sugar nucleotides are considered to be formed by transfer of a nucleotidyl group to an aldose 1-phosphate. The nucleotidyl donor may be a nucleotide triphosphate, as in the formation of the uridine diphosphate D-glucose from uridine triphosphate and α-D-glucose 1-phosphate; or another sugar nucleotide, as in the formation of uridine diphosphate D-galactose from uridine diphosphate D-glucose and α-D-galactose 1-phosphate.

The sugar moiety of the nucleotide may undergo a number of enzymic reactions, forming various

derivatives. The latter may be oxidized to uronic acids and decarboxylated to a pentose, or epimerized to another isomeric monosaccharide. The transfer of these sugar moieties by various enzymes to appropriate acceptors will result in the formation of diverse complex saccharides, such as glycogen, chitin and a number of others.

The Structure of Proteins

By M. F. Perutz. (*Medical Research Council Unit for Molecular Biology, Cavendish Laboratory, Cambridge*)

The structure of proteins presents a great challenge to X-ray crystallography. It is the only method which is in principle capable of solving this problem, yet the enormous amount of information provided by the X-ray diffraction pattern of a protein crystal cannot be interpreted without knowing the phases of the diffracted rays. These can be measured only if an isomorphous series of crystals is available, one member of the series containing the pure protein, and the others compounds of the protein with heavy atoms attached to specific sites (Green, Ingram & Perutz, 1954).

Two structures have recently been solved by this method. Kendrew and his collaborators first obtained a three-dimensional picture of sperm-whale myoglobin at a limited resolution of 6Å (Kendrew *et al.* 1958) and later raised the resolution to 2Å (Kendrew *et al.* 1960). The author and his colleagues have obtained a three-dimensional picture of horse haemoglobin at 5·5Å resolution (Perutz *et al.* 1960).

At a resolution of 5·5 or 6Å, the results show the position of the haem group and the general lay-out of the polypeptide chain, usually referred to as the tertiary structure. At a resolution of 2Å the folding of the polypeptide chain is revealed in detail and many of the side chains can be identified, making it possible to construct an atomic model of the protein.

The structures of the two proteins were described and their relation to the amino acid sequence of human haemoglobin (Braunitzer, 1961) was discussed.

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Protein Structure in Relation to Enzymic Activity, with Special Reference to Papain

By E. L. Smith (with A. Light and J. R. Kimmel). (*University of Utah College of Medicine, Salt Lake City, Utah, U.S.A.*)

Evidence has been presented by Hill & Smith (1960) that crystalline mercuripapain can be degraded by leucine aminopeptidase to an enzymically active fragment which contains approximately 76 residues and which should represent the carboxyl terminal portion of the papain molecule. Structural studies of intact papain currently in progress have permitted the tentative elucidation of the sequence of amino acids in this portion of the molecule. This has been accomplished by isolation of peptides from oxidized, *S*-carboxymethylated and denatured papain which were digested with chymotrypsin, pepsin or trypsin. Peptides were isolated by ion-exchange and partition chromatography, as well as by zone electrophoresis. Amino acid sequences were determined by chemical and enzymic methods. Comparison of the composition of the *C*-terminal 74 residues of papain with the average composition of the fragment are in satisfactory agreement. Certain features of the *C*-terminal 74 residues are of particular interest. The amino terminal sequence of this portion of the molecule is ...Gly.Pro.CyS.Gly... where the half-cystine residue is part of a disulphide bridge; this type of sequence might be expected to offer considerable resistance to hydrolysis by the aminopeptidase. From studies of inhibition, chemical reactivity and kinetic behaviour with simple substrates, active papain contains a single reactive thiol group. It is also known from kinetic evidence that a carboxylate ion is concerned in the enzymic activity [see Smith (1958) and review by Smith & Kimmel (1960)]. The terminal region of 74 residues contains three half-cystine residues corresponding to one disulphide bridge and one thiol group. Tentative identification of the bridge has been made by isolating from a peptic digest of denatured papain two peptides of known sequence linked by a disulphide bridge. It is noteworthy that the 74-residue structure contains 3 aspartyl and 3 glutamyl residues (in addition to amides of these residues) with an aspartyl residue on either side of what is, apparently, the active thiol group. It is well known that the best substrates for papain are compounds which possess arginyl or lysyl residues

and that compounds which have ionized carboxyl groups are poor substrates for the enzyme. The proximity of two ionized carboxyl groups near the active thiol could aid in explaining the side-chain specificity since electrostatic factors would play a major role in attracting cationic groups and repelling anionic ones. Inasmuch as the two half-cystine residues of the 74-residue sequence are separated by 59 residues, the disulphide bridge must play a major role in helping to maintain a tightly folded structure. However, other forces are certainly involved since papain and the active fragment are sensitive to denaturation by urea, guanidine salts and exposure to acid. Portions of the amino acid sequence show regions of high net charge as well as regions devoid of charge but possessing many hydrophobic side-chains.

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Control of Adaptive Enzyme Synthesis

By J. Monod. (*Institut Pasteur, Paris, XV, France*)

The structure of enzyme proteins, including adaptive enzymes, appears to be entirely controlled by specific genetic determinants (structural genes). The rate at which enzymes are synthesized under the control of structural genes is determined by other genes (regulator genes) which govern the synthesis of intracellular repressors. The effects of small-molecular-weight substances in inducing or inhibiting the synthesis of certain enzymes is due to an interaction with these genetically determined repressor substances. Different lines of evidence indicate that the repressors operate directly at the genetic level by inhibiting the activity of structural genes. This leads in turn to distinctive predictions concerning the properties of the intermediates through which structural information is conveyed from the genes to the cytoplasmic enzyme-forming centres. A ribonucleic acid fraction possessing the required properties has been shown to exist in bacterial cells. Preliminary experiments confirmed the hypothesis that this fraction serves as a 'messenger' between the structural genes and the ribosomes where the synthesis of enzyme takes place.

COMMUNICATIONS

(1) A Case of Limit Dextrinosis

By D. J. Manners and A. Wright. (*Department of Chemistry, University of Edinburgh*)

Several types of glycogen-storage disease (glycogenosis) are now known; in some of these, the accumulation of glycogen is caused by a relative deficiency of one of the enzymes concerned with glycogenolysis (Cori, 1952–53; Stetten & Stetten, 1960). In cases of limit dextrinosis (type 3 glycogenosis), the glycogen has an abnormal structure with short exterior chains, and a deficiency of the 'debranching' enzyme-system is indicated.

Glycogen from a liver biopsy sample (233 mg. of tissue from a girl aged 18 months) has been analysed. By the Pflüger technique, 34 mg. of polysaccharide was obtained, corresponding to a tissue content of 15%. Acid hydrolysis gave only glucose. The polysaccharide was characterized as a glycogen since it had an appreciable iodine-staining power ($\lambda_{max.}$ 440 mμ) which was destroyed by incubation with salivary α-amylase. The average chain length, determined by quantitative α-amylolysis, was 8 glucose residues. Since the β-amylolysis limit was 27%, the exterior and interior chains contained 4–5 and 2–3 glucose residues respectively (compare values of about 8 and 3 for a normal glycogen). The glycogen thus resembles a muscle-phosphorylase limit dextrin.

Several enzymes are concerned in the complete degradation of glycogen into α-D-glucosyl phosphate, including phosphorylase, trans-α-glucosylase and amylo-1:6-glucosidase (Walker & Whelan, 1960). A deficiency of either trans-α-glucosylase or amylo-1:6-glucosidase, or of both enzymes, will prevent the hydrolysis of α-1:6-glucosidic inter-chain linkages, and hence, glycogen breakdown will be incomplete.

It is suggested that three types of limit dextrinosis may exist; type 3a, caused by a lack of trans-α-glucosylase, type 3b, by a deficiency of amylo-1:6-glucosidase, and type 3c in which the activity of both enzymes is reduced. In types 3a and 3c the exterior chains in the residual glycogen would contain four glucose residues whereas in type 3b, the combined action of phosphorylase and trans-

α-glucosylase would reduce the exterior chains to only one, two or perhaps three glucose residues.

The biopsy glycogen differs significantly from a limit dextrinosis glycogen (average chain length, 6 glucose residues; β-amylolysis limit, 14%) examined previously (Manners, 1954). The former may therefore be a type 3a or 3c disease, and the latter a type 3b.

We thank Dr A. D. Bain and Dr D. Douglas, The Royal Hospital for Sick Children, Edinburgh, for their collaboration, and Professor E. L. Hirst, F.R.S., for his interest in this work.

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(2) The Structure of Phosphomaltotetraose

By F. W. Parrish* and W. J. Whelan. (*The Lister Institute of Preventive Medicine, London, S.W.* 1)

Potato starch contains a small proportion of glucose residues substituted at C-6 by phosphate (Posternak, 1935). These units are mainly in the amylopectin component (Samec, 1914; Schoch, 1942). α-Amylolysis of potato starch yields a stable phosphorylated limit dextrin, maltotetraose monophosphate (phosphomaltotetraose), which Posternak (1951) found to contain the phosphate group on one or other of the internal glucose units. This communication describes the precise location of the phosphate group.

The experimental procedure, which requires as little as 2 mg. of material, combined two techniques of periodate oxidation recently used to explore the structures of amylaceous oligo- and poly-saccharides. (1) The erosive over-oxidation by periodate of α-1:4-linked glucosaccharides commences at the reducing chain end and ceases when any substituent other than at the 1:4-positions is encountered (Whelan & Bines, 1955; Hough & Perry, 1956; Duncan & Manners, 1958; Bines & Whelan, 1960). (2) The rate of the erosion depends on the proportion of reducing chain ends, and hence the size of any amylosaccharide can be determined by comparing its rate of oxidation, measured by liberation of formaldehyde, with that of a structurally similar compound of known size (Parrish & Whelan, 1959).

Three samples of Posternak's (1951) phosphomaltotetraose were isolated from potato starch by digestion with crude saliva and crystalline salivary α-amylase. All behaved in the same manner in the following respects. The molar ratio of phosphorus to glucose units was 4·0. The rate of periodate oxidation was 1·25 times that of maltopentaose compared in terms of glucose content, and the dextrin was therefore a tetrasaccharide, confirming Posternak's (1951) observation. The molar ratio of formaldehyde was 1·80 molecular proportions, equal to that formed from maltose (calc. 2 mol. prop.). The phosphate group is therefore located on the third glucose unit from the reducing end of maltotetraose and the phosphodextrin may be described as maltotetraose 6^3-phosphate (nomenclature as by Whelan, 1960).

The phosphodextrin, which is the smallest formed by salivary α-amylase, bears a formal resemblance to the α-limit dextrins of amylopectin, which owe their resistance to attack to steric inhibition by 1:6-linkages. The smallest of these α-limit dextrins, formed at different stages of α-amylolysis (Walker & Whelan, 1960), are 6^3-α-glucosylmaltotriose (Nordin & French, 1958) and 6^3-α-maltosylmaltotriose (Bines & Whelan, 1960).

The periodate erosion method is general in its applicability to substituted maltodextrins and has been used with limit dextrins formed by other α-amylases (Hughes, 1960).

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(3) The Fractionation of Human Cartilage Chondromucoprotein

By A. J. Anderson. (*Department of Pathology, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex*)

A chondromucoprotein fraction (fraction A), extracted from human cartilage at pH 10·6

(Anderson, 1961), has been chromatographed on diethylaminoethylcellulose (DEAE-cellulose) using buffer mixtures of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) and citrate. About 30% of fraction A did not adsorb at pH 8·6 (5 mM-citrate–tris). This unadsorbed fraction (subfraction A) was highly viscous [as judged by the flow time of an aqueous 0·5% (w/v) solution in an Ostwald viscometer]. Subfraction B was obtained by elution with 20 mM-citrate–tris, pH 7·4. Three further citrate–tris buffers (50 mM, pH 6·1), containing 0·3, 0·7 and 1·0M-NaCl, yielded subfractions C, D and E respectively. Subsequent extraction of the DEAE-cellulose with alkali at pH 11·7 yielded subfraction F. Analysis (subfractions A and D respectively): N, 8·4, 5·6%; sialic acid [by the thiobarbituric acid method of Warren (1959)], 2·1, 1·4%; hexuronide, 8·7, 18·1%; hexosamine, 14·0, 18·8%; protein (calculated from total N after subtraction of sialic acid and hexosamine N), 45, 25%. Subfractions A and D contained 72 and 93% of the total sialic acid and hexuronide respectively, eluted from the column.

Appreciable amounts of sialic acid and protein in subfractions B, C, E and F, accompanied by only traces of hexuronide, indicates the presence in human chondromucoprotein of glycoprotein and protein material unassociated with mucopolysaccharides. Subfractions B to F had viscosities comparable to water and would appear to be low viscosity components of fraction A.

Recent work has shown that injection of papain and human plasmin into rabbits leads to breakdown of the mucopolysaccharide–protein complexes (chondromucoprotein) in cartilage, with subsequent appearance of chondroitin sulphate in blood and urine (Spicer & Bryant, 1958; Lack, Anderson & Ali, 1961). The following enzymes were found to degrade subfraction A as judged by viscosity decreases: papain (0·5, 82); trypsin (1·0, 67); testicular hyaluronidase (46, 72); streptokinase-activated human plasminogen (3·0, 34). The first figure in brackets refers to the time (hours), and the second figure the maximum decrease in flow time (%) following enzyme addition. None of these enzymes resulted in the release of significant amounts of sialic acid. The quantitative release of sialic acid following incubation of subfraction A with receptor-destroying enzyme (from a *Vibrio cholerae* filtrate) was not accompanied by a significant drop in viscosity.

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(4) The Development of Invertase Activity in Washed Slices of Root Tissue

By J. S. D. Bacon. (*Department of Biochemistry, Macaulay Institute, Aberdeen*)

MacDonald & DeKock (1958) found that sucrose was rapidly converted into monosaccharide when disks cut from sugar-beet root were washed in aerated running tap water; the formation of a trisaccharide led them to infer that invertase was responsible. However, the invertase content of this root is known to be very low (Allen & Bacon, 1956).

We have investigated this phenomenon, using the following procedure to measure invertase activity: disks [1 cm. diam. and about 1 mm. thick, each weighing about 0·1 g., cut as described by MacDonald & Knight (1958)] were placed in cold ethyl acetate for 15 min., then shaken in a mixture of ice, water, and ethyl acetate for 20 min., and finally for 10 min. in cold water. This removes much of the cell contents, including the sugars. Treated disks (20) were then immersed in 22 ml. of sucrose solution (buffered with sodium acetate to pH 5·0 and containing 1·2 g. of sucrose) and shaken at 35°. After 30 min. a 10-ml. sample was withdrawn and pipetted into 10 ml. of 5% Na_2CO_3 solution; after a further 2 hr. a second sample was similarly treated. From the change in optical rotation the hydrolysis of sucrose was calculated (cf. Burger, Bacon & Bacon, 1961).

No evidence was found for loss of invertase from the disks during the preliminary treatment. Untreated disks had the same invertase activity; the cell contents removed by washing after ethyl acetate treatment had a negligible amount.

When tested in this way freshly cut disks had no measurable invertase activity, although considerably prolonged incubation (1–5 days) revealed that some was present. After 1 day's washing at 25° in the apparatus described by McDonald & Knight (1958) activity of the order of 1–5 mg. of sucrose hydrolysed/g. fresh weight/hr. was detectable, and after 3 or 4 days the activity reached a maximum of 15–40 mg./g./hr., which was thereafter maintained until the disks became depleted of soluble carbohydrate and began to disintegrate (8–10 days).

The invertase had the transferase characteristics of a plant invertase, and the amount developed was more than sufficient to bring about the observed hydrolysis of the relatively large amounts of sucrose present in sugar-beet root.

A similar development of invertase activity was found in root tissue from Composites, in which the water-soluble carbohydrate fraction is more complex.

The author is grateful to Dr I. R. MacDonald and Mr A. H. Knight, of the Plant Physiology Department of this Institute, for preparing the washed disks.

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(5) Production of a Strong β-Glucuronidase Inhibitor in Mammalian Tissues

By C. A. Marsh. (*Rowett Research Institute, Bucksburn, Aberdeen*)

The existence in urine of β-glucuronidase inhibitors of unknown composition has long been recognized (see Abul-Fadl, 1957). An examination of a dialysate from normal human urine revealed the presence of an inhibitor, the potency of which was enhanced by acid treatment and decreased after treatment with alkali. Both treatments were reversible. The properties are consistent with those of the known specific inhibitors D-glucaro-1:4-lactone and galactarolactone.

The presence of such compounds in mammalian tissues has not been reported, but D-glucaro-1:4-lactone could conceivably arise by oxidation of D-glucuronic acid or of its 6:3-lactone. The former is a feeble inhibitor of β-glucuronidase, whereas the latter is non-inhibitory. D-Glucuronic acid may be formed *in vivo* through the breakdown of uridine diphosphate glucuronic acid or other natural derivatives of D-glucuronic acid, and may participate in mammalian ascorbic acid synthesis.

Rat-liver homogenates were found to convert D-glucurono-6:3-lactone into a product which inhibited rat-liver β-glucuronidase. The inhibitory power was greatly increased by acid treatment and reversibly abolished by treatment with alkali, consistent with the production of an inhibitory lactone. The enzymic activity was largely confined to the supernatant from the high-speed centrifuging of an isotonic homogenate. Partially purified preparations required the addition of diphosphopyridine nucleotide, and the latter potentiated crude preparations. D-Glucuronic acid was much less effective as a precursor of the inhibitor, and L-gulono-1:4-lactone and ascorbic acid were ineffective. Inhibitor formation was reduced by addition of veronal.

Ingestion of D-glucurono-6:3-lactone increased greatly the production of urine inhibitor, whilst Flaschenträger, Cagianut & Meier (1945) reported increased excretion of furan-2:5-dicarboxylic acid which could have been derived from D-glucaric acid by their method of isolation.

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(6) An Enzymic Method for the Estimation of Small Quantities of Glutamic Acid

By Julia M. Sowerby and J. H. Ottaway. (*Department of Biochemistry, University of Edinburgh*)

Ells (1959) has described a method of assaying the activity of lactic dehydrogenase, coupling the oxidation of reduced diphosphopyridine nucleotide (DPNH) to the reduction of 2:6-dichlorophenol-indophenol by means of phenazine methosulphate. The principle has been adapted to the measurement of small quantities ($< 1\,\mu$mole) of glutamic acid, using a Na_2SO_4 suspension of glutamic dehydrogenase (Boehringer). It was found easier to get a linear response between dye reduction and glutamate concentration when a tetrazolium salt, rather than indophenol, was used as hydrogen acceptor. 3-*p*-Iodophenyl-2-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) (British Drug Houses Ltd.) was reduced satisfactorily in this system (Nachlas, Marguilies & Seligman, 1960).

The reaction is carried out in 0·06M-2-amino-2-hydroxymethylpropane-1:3-diol(tris)–HCl buffer, pH 7·6. The reaction mixture contains 1·12 mM-INT, 17·5 μM-phenazine methosulphate, 0·465 mM-DPNH, 6 mM-KCN, approx. 0·5 mM-ethylenediaminetetra-acetic acid (EDTA), 200 μg. of glutamic dehydrogenase and 0–0·5 μmole of glutamate, in a final volume of 3 ml. The enzyme and DPN are added last; glutamic dehydrogenase is stable in dilute solution if the solution is buffered to around pH 7 and contains at least 0·2 mM-DPNH (Frieden, 1959). A suitable volume of glutamic dehydrogenase suspension is therefore diluted each day to a concentration of 400 μg. of protein/ml., with 0·09M-tris–HCl buffer, pH 7·6, containing 3 mM-DPNH and approx. 3 mM-EDTA. This solution is stable for at least 24 hr. at 1°, and for extended periods at room temperature.

The mixture is incubated at 37° for 20 min. (or longer) and the formazan is then dissolved by the addition of 5 ml. of acetone. Removal of enzyme protein is not necessary; the extinction of the solution may be read at 460 mμ against a reagent blank. The colour is stable for at least 12 hr. in the

dark and 3 hr. in light. The response is linear up to 0·3 μmoles of glutamate, and values are reproducible from day to day.

Ammonium ions in large excess inhibit colour development and make the response non-linear, but in concentrations up to 0·6 mM (2 μmoles/sample) the effect is not appreciable.

Saturated $KClO_4$ inhibits the enzyme, and trichloroacetic acid forms an insoluble salt with INT, but deproteinization of samples with 50% ethanol–N-HCl appears to be satisfactory.

Values for glutamic acid content of animal tissues were presented.

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(7) The Pyridine Nucleotide Specificity of *iso*Citric Dehydrogenase in Plant Mitochondria

By J. M. Tager. (*Margaretha Mes Institute for Plant Physiology and Biochemistry, University of Pretoria, South Africa*)

Two *iso*citric dehydrogenases, one specific for diphosphopyridine nucleotide (DPN) and the other for triphosphopyridine nucleotide (TPN), have been extracted from yeast (Kornberg & Pricer, 1951), from particulate preparations obtained from pigeon-breast muscle, guinea-pig heart and ox heart (Plaut & Jung, 1954) and from pea-seedling mitochondria (Davies, 1955). The activity of the DPN-specific *iso*citric dehydrogenase is high in Ehrlich ascites cell mitochondria (Hawtrey & Silk, 1960) and low (Plaut & Jung, 1954; Ernster & Navazio, 1957) or absent (Purvis, 1958) in rat-liver mitochondria.

The pyridine nucleotide specificity of *iso*citric dehydrogenase in mitochondria isolated from ripe fruits of the papaya (*Carica papaya* L.) was studied. When 2:6-dichlorophenolindophenol, ferricytochrome *c* or O_2 was used as electron acceptor, these mitochondria oxidized *iso*citrate at a negligible rate unless DPN was added. TPN promoted *iso*citrate oxidation to a slight extent and the rate with DPN and TPN was equal to the rate with DPN alone. When mitochondria were incubated with *iso*citrate, KCN and DPN, an increase in optical density at 340 mμ was observed which was greatly enhanced by the addition of amytal. In similar tests with TPN, only a slight increase in optical density at 340 mμ occurred. The mitochondria catalysed a very rapid reduction of 2:6-dichlorophenolindophenol or ferricytochrome *c* by reduced DPN and a less rapid reduction by reduced TPN. The rate of reduction of these oxidants by reduced TPN was not enhanced by DPN.

These results indicate that papaya mitochondria contain a DPN-specific *iso*citric dehydrogenase and that the mitochondrial oxidation of isocitrate is not mediated by a TPN-specific *iso*citric dehydrogenase linked to DPN through a transhydrogenase.

Similar results were obtained with mitochondria isolated from a variety of plant tissues including storage organs, flower buds, leaves, green and ripe fruits and seedling tissues. Mitochondria isolated from these tissues all appear to contain a DPN-specific *iso*citric dehydrogenase and in most cases the TPN-specific isocitric dehydrogenase is predominantly extra-mitochondrial.

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(8) Lactic Dehydrogenases of *Lactobacillus arabinosus*

By A. M. Snoswell. (*Department of Biochemistry, University of Adelaide, South Australia*)

The partial purification of a lactic dehydrogenase of *Lactobacillus arabinosus* which was specific for L-lactic acid and independent of diphosphopyridine nucleotide (DPN) was previously reported (Snoswell, 1959*a*). Evidence suggested that this enzyme was a flavoprotein. Subsequently a D- and an L-lactic dehydrogenase were isolated from cell-free extracts by a procedure involving column chromatography on cellulose ion-exchange resins (Snoswell, 1959*b*). Both these enzymes were DPN-independent. Later Dennis & Kaplan (1960) reported the partial purification of two stereospecific lactic dehydrogenases from *Lactobacillus plantarum* (*L. arabinosus*) which were DPN-dependent.

A study of the properties of the two pairs of enzymes was undertaken and revealed the following differences. The optimum pH values for the DPN-independent D- and L-lactic dehydrogenases are 6·7 and 6·0 respectively while those reported

for the DPN-dependent enzymes are 8·5 and 7·5 respectively. The DPN-independent D-lactic dehydrogenase has a greater affinity for substrate than the L-lactic dehydrogenase as evidenced by the lower K_m values of the former enzyme, regardless of the nature of the hydrogen acceptor; the opposite was reported for the DPN-dependent enzymes. Further, the two DPN-independent enzymes are readily separated by electrophoresis whereas Dennis & Kaplan (1960) could not separate the DPN-dependent enzymes even after prolonged electrophoresis. Finally the two DPN-dependent enzymes were completely separated from the DPN-independent enzymes on a triethylaminoethyl(TEAE)-cellulose column by eluting with sodium lactate.

The DPN-independent L-lactic dehydrogenase was purified some 1200-fold by further column chromatography on TEAE- and ECTEOLA-cellulose (Peterson & Sober, 1956) columns. The final preparation was yellow-brown in colour and contained flavin solely in the form of flavin mononucleotide in an amount equivalent to one molecule of mononucleotide per molecule of enzyme. The flavin was reduced approx. 20% by the addition of L-sodium lactate and the difference spectrum showed that the flavin absorption maxima at 370 and 455 mμ were reduced, with the concurrent appearance of a broad absorption band having a maximum at approx. 570 mμ.

Thus *L. arabinosus* contains two pairs of stereospecific lactic dehydrogenases; two are DPN-dependent whereas the other two are DPN-independent and are probably both flavoproteins.

This work was supported by a grant from the Australian National Health and Medical Research Council.

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(9) Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation

By P. Mitchell. (*Zoology Department, University of Edinburgh*)

The concept of group translocation—the vectorial movement of chemical groups during group transfer (Mitchell, 1957, 1959)—leads to the idea that the chemical reactions catalysed by two enzymes that translocate a common component will be coupled osmotically when this component passes from the one active centre to the other through a closed osmotic feature, such as a membrane-limited compartment (Mitchell & Moyle 1958*a*, *b*). Although this type of conception is latent in work on ion transport and respiration (see Robertson, 1960), the translocation feature of chemiosmotic coupling has made it elusive to explicit description in the scalar idiom of biochemistry. The author proposes, therefore, to define explicitly a chemiosmotic hypothesis of electron-transport phosphorylation (Mitchell, 1960), as a basis for extension or disproof.

(i) Electron transfer, driven by oxido-reduction or photons, occurs vectorially across a membrane, separating aqueous phases A and B.

(ii) Process (i) effectively generates H^+ in A and OH^- in B.

(iii) The membrane is relatively impermeable to ions, but may allow exchange (Ussing, 1947) of H^+ and/or OH^- against ions of equivalent and like charge. The skew of $[H^+]$ ([] denoting electrochemical activity) therefore shows as a pH difference (pH_{B-A}) plus a membrane potential (mv_{A-B}). Approximately,

$$[H^+]_A/[H^+]_B = 10^{pH_{B-A}} \times 10^{mv_{A-B}/60} \leqslant e^{-(\text{work per electron translocated}/kT)}$$

(iv) The membrane contains an anisotropic adenosine triphosphatase system (phosphate-accepting active centre, E) catalysing the reaction: phosphate + ADP $\rightleftharpoons$ ATP + H^+ + OH^-.

(v) E communicates rapidly with OH^- of A and H^+ of B, but slowly with H^+ of A, OH^- of B, and H_2O of A and B. Consequently,

$$[H_2O]_{A \text{ or } B} \geqslant [H_2O]_E \geqslant ([H_2O]_{A \text{ or } B} \times [H^+]_B)/[H^+]_A,$$

when $$[H^+]_B/[H^+]_A < 1.$$

The inequalities of (iii) and (v) depend upon 'leakiness' and show as uncoupling. The H^+ differential, generated by electron translocation, dehydrates phosphate + ADP (or other acidic acceptor) by withdrawing OH^- and H^+ from phosphorylium and acidic acceptor respectively along different, chemically specific, translocation paths in the adenosine triphosphatase system. Using (iii), (v), and equilibrium constant data (Atkinson, Johnson & Morton, 1959): at 10 mM-inorganic phosphate, [ATP]/[ADP] would be about unity if, for example, A were 2 pH units below and 300 mv above B, and the system were well coupled.

The accepted maximum P/O quotients are consistent with the chemiosmotic coupling hypothesis. This hypothesis explicitly recognizes the vectorial character of the catalysis and so can account directly for the uncoupling effect of substances or treatments that homogenize or loosen the catalytic system.

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(10) Discrepancies in the Published Intracellular Distribution of Aldolase in Brain Homogenates

By M. K. Johnson. (*Toxicology Research Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey*)

Hexokinase has been shown to be the only glycolytic enzyme localized chiefly in the mitochondrial fraction of rat-brain homogenates prepared in 0·3M-sucrose (Johnson, 1960). However, it has been claimed (Brunngraber & Abood, 1960) that brain mitochondria stimulate anaerobic production of lactate by brain supernatant in a medium containing a limited quantity of fructose 1:6-diphosphate, and that this is due to aldolase being predominantly present in the mitochondria. The intracellular distribution of aldolase has therefore been determined in more detail, and compared with the distribution of glycolytic activity measured in a more rational medium containing ample fructose 1:6-diphosphate. Of the total homogenate activity, about 20% was found in washed mitochondrial fractions in the cases of lactic dehydrogenase, aldolase, and of the system converting fructose 1:6-diphosphate into lactate. This was reduced to about 10% in each case if a Nalco blender was used instead of a smooth high-speed Perspex pestle homogenizer (Aldridge, Emery & Street, 1960); 65–70% of the total was found in a crude supernatant fraction (20 000*g*, 30 min.). About 6% stimulation of lactate production usually occurred when mitochondrial preparations were mixed with supernatant. This is hardly significant, but may reflect a slight disproportionation in distribution of some of the soluble enzymes due to differing mild degrees of adsorption on particulate matter; such small variations have been shown previously (Johnson, 1960). Glycolytic activity of all fractions was somewhat depressed (17%) by addition of pyruvate. The mean activity of a mixture of equivalent amounts of mitochondria and supernatant was 35·5 mg. of lactic acid/g. of wet brain/30 min. at 37°, pH 7·2 in the absence of pyruvate.

The glycolytic medium used by Brunngraber & Abood (1960) is clearly unsuitable in that supplies of fructose 1:6-diphosphate are inadequate to maintain the reaction throughout the whole period, so that a dependence on added glucose develops. This complicates the study by involving the hexokinase reaction and a balance of adenosine tri- and di-phosphates, but cannot fully explain the discrepancy. The possibility is examined that soluble aldolase is adsorbed on the surface of particles when homogenization is carried out in sucrose-containing unbuffered ethylenediaminetetra-acetic acid.

Lactate production is not proportional to the amount of tissue taken in the range used by Brunngraber & Abood (1960); this gives erroneous results, including an apparent stimulation when fractions are mixed.

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(11) Free Amino Acids in the Intracellular Osmoregulation of Euryhaline Marine Worms

By Ch. Jeuniaux, Gh. Duchâteau-Bosson and M. Florkin. (*Department of Biochemistry, University of Liège, Belgium*)

Duchâteau, Jeuniaux & Florkin (1961) have pointed out that, in more or less euryhaline marine invertebrates, the degree of euryhalinity is accounted for, either by an adjustment of the intracellular concentration, a regulation in which free amino acids are involved in all cases studied so far, or by a combination of an osmoregulation of the blood and of an adjustment of the intracellular concentration to the level maintained in the blood as a result of osmoregulation. Cases in the first category are represented by *Mytilus* (Potts, 1958) and *Arenicola marina* (Duchâteau *et al.* 1961). Among the forms belonging to the second category, we find *Carcinus maenas* (Duchâteau & Florkin, 1956; Shaw, 1958) and *Eriocheir sinensis* (Duchâteau & Florkin, 1955). The authors have shown that the situation prevailing in *Arenicola* is also present in *Perinereis cultrifera*, a marine annelid showing a limited degree of euryhalinity. This species has no osmoregulation of the blood but is able to adjust the intracellular concentration, a process in which the variation of concentration of the free amino acids plays a role. A much more euryhaline annelid, *Nereis diversicolor*, relies on

osmoregulation of the blood and on an intracellular regulation realized at least partly by a marked change of concentration of intracellular free amino acids. The low degree of tissue hydration which is observed in diluted media accounts only for a small percentage of the change observed in the concentration of the free amino acids.

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(12) Further Observations on the Nature of the Diauxic Effect with *Pseudomonas aeruginosa*

By W. A. Hamilton and E. A. Dawes. (*Department of Biochemistry, University of Glasgow*)

We have shown previously that when cells of *Pseudomonas aeruginosa*, grown in mineral-salt medium with an organic acid as sole source of carbon and energy, are inoculated into a medium containing glucose and the organic acid ('mixed medium'), diauxic growth occurs with the acid as growth substrate during the initial phase. This behaviour extends to non-proliferating suspensions. Citrate-grown cells which have been induced with glucose in washed suspension do not metabolize glucose in the presence of an equimolar concentration of citrate until the citrate concentration has fallen to a low level (Hamilton & Dawes, 1959). It was found that the intracellular enzymes of glucose metabolism in this organism are inducible, and the possible role of a glucose permease in these diauxic effects was also discussed (Hamilton & Dawes, 1960).

The inhibition of glucose metabolism by the simultaneous oxidation of citrate has been demonstrated in cell-free extracts. The extracts oxidize glucose only by the direct pathway to 2-oxogluconate, and the inhibitory effect produced by citrate metabolism on the activity of glucose dehydrogenase persists after the added citrate has been utilized.

A study of growth with inoculum cells which have been grown on glucose instead of citrate shows that in the mixed medium the culture continues to synthesize the glucose enzymes. Although two different growth rates are discernible, both glucose and citrate are metabolized simultaneously and a characteristic diauxic picture is not obtained. Cohn & Horibata (1959) have described such maintenance of synthesis of induced enzymes and have explained it in terms of a permease.

Citrate did not inhibit glucose metabolism with cells harvested from this mixed medium, in contrast with cells grown on citrate and induced with glucose in washed suspension.

Further indication of a glucose permease was obtained in experiments which showed an increased fragility of protoplast-like structures, prepared from citrate-grown cells, in a glucose stabilizing medium subsequent to induction with glucose.

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(13) Metabolism of 4-Chloro-2-methylphenoxyacetic Acid by a Soil Micro-organism

By J. K. Gaunt and W. C. Evans. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

A gram-negative soil bacterium has been isolated, capable of utilizing the herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA), up to a concentration of 0·1%, as sole source of organic carbon, when grown aerobically in a mineral–salts medium. During growth, the concentration of MCPA falls off rapidly, and the u.v. maxima of MCPA (228 and 279 mμ) are replaced by a higher peak at 277 mμ.

Culture medium (50 l.) was extracted at a point when no MCPA remained but a strong u.v. absorption at 277 mμ was still present. From the acid ether extract a white crystalline product (1 g.) was isolated, m.p. 181–183°, $\lambda_{max.}$ 277 mμ (in water) changing to $\lambda_{max.}$ 250 mμ in alkaline solution. No halogen was present but the hydroxamic acid test was positive (Found: C, 54·91, 54·71; H, 3·92, 4·07. $C_7H_6O_4$ requires C, 54·50; H, 3·90%). Microtitration indicated a monobasic acid, equiv. wt. 157; hydrolysis in aqueous solution liberated a second carboxyl group. The solution now gave an immediate Rothera reaction. These properties are consistent with the substance being the lactonic acid, α-methyl-γ-carboxymethylene-Δ^{α}-butenolide (previously undescribed). Peracetic acid oxidation of 5-chloro-*o*-cresol (5-chloro-2-hydroxytoluene) yielded a halogen-free crystalline product, m.p. 182°, identical with the culture acid (mixed m.p. undepressed).

MCPA cultures transiently give a Folin reaction. From 50 l. of culture medium, a halogen-containing phenolic acid (2 mg.) was isolated, m.p. 98°; $\lambda_{max.}$ 276 mμ in acid, 293 mμ in alkali. Microtitration indicated a monobasic acid, equivalent weight 216; calculated for 6-hydroxy-MCPA ($C_9H_9O_4Cl$) 216·5. Chromatographic and u.v. spectra characteristics indicate that MCPA and this compound are related in a way analogous to the resemblance between 2:4-dichlorophenoxyacetic acid and its 6-hydroxy derivative. Proof must await an unambiguous synthesis of 6-hydroxy-MCPA.

A neutral Folin-positive substance in extracts has been identified as 5-chloro-*o*-cresol by chromatography and u.v. spectroscopy.

Continuous ether extraction of the residual culture medium gave a further compound (400 mg.) which proved to be oxalic acid (mixed m.p. undepressed).

Isolation of these presumed intermediates suggests the following pathway for the microbial metabolism of MCPA: MCPA → 6-hydroxy-MCPA → 5-chloro-3-methylcatechol → α-methyl-γ-chloromuconic acid → α-methyl-γ-carboxymethylene-Δ^{α}-butenolide → α-methylmaleyl acetate → smaller fragments prior to entering a terminal respiratory cycle. None of these steps have yet been attempted enzymically.

The relevance of 5-chloro-*o*-cresol and oxalic acid in the metabolic pathway has not been fully investigated.

One of us (J.K.G.) is grateful to the Ministry of Agriculture, Fisheries and Food for a post-graduate scholarship.

(14) The Amino Acid Sequence around Serine Phosphate in Phosphoglucomutase from Different Origins

By C. Milstein.* (*Department of Biochemistry, University of Cambridge*)

The sequence around the serine phosphate of rabbit-skeletal-muscle phosphoglucomutase has recently been elucidated (Milstein & Sanger, 1961) as Thr.Ala.SerP.His.Asp. Crystalline enzyme was used in these studies. A study of the sequence around the serine phosphate of the phosphoglucomutase of yeast and rat liver, using only partially purified preparations, is described in this paper.

Yeast phosphoglucomutase was prepared as by McCoy & Najjar (1959) and the fraction following ammonium sulphate precipitation was used. Rat-liver phosphoglucomutase was made by an unpublished procedure involving ammonium sulphate fractionation and column chromatography on diethylaminoethylcellulose.

The method of labelling the enzymes was the same as that used previously for the muscle phosphoglucomutase (Milstein & Sanger, 1961). The approximate radioactivity of the preparations was 2·5 μmc/mg. of protein for the rat enzyme and 12·5 μmc/mg. for the yeast enzyme. Considerable radioactivity is released after precipitation of the proteins by ammonium sulphate; most of that remaining is released on incubation with glucose 1-phosphate, suggesting that the labelled protein is phosphoglucomutase.

The labelled proteins thus prepared were subjected to partial acid hydrolysis and then to ionophoresis at pH 3·5. Phosphoglucomutase from rabbit muscle was subjected to the same treatment and used for comparison. Subsequent radioautography, showing perfect matching of all the bands of the hydrolysates of the three preparations, strongly suggests that the same pentapeptide is present in all three cases.

The radioactive proteins were also digested with trypsin. The pattern of the tryptic peptides differ in the three cases. The main radioactive peptides obtained were subjected to partial acid hydrolysis and then to ionophoresis at pH 6·5. The three showed the same pattern, also supporting the suggestion that the pentapeptide Thr.Ala.SerP.His.Asp is common to the phosphoglucomutases of rabbit muscle, rat liver and baker's yeast.

The possibility exists that some SerP peptides have the same electrophoretic mobility but different amino acid compositions, and thus are not detected by this method. This is, however, unlikely, due to the proportions of the bands present. In particular, the dipeptide SerP.His is present in the three enzymes since histidine is the only basic residue changing its mobility below pH 6·5.

The author is indebted to Dr F. Sanger, F.R.S., for invaluable discussions and to the Medical Research Council for a grant.

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* On leave from the Instituto Nacional de Microbiologia, Buenos Aires, Argentina.

(15) The Molecular Composition of Haemoglobins A_2 and G_2

By E. R. Huehns* and E. M. Shooter. (*Department of Biochemistry, University College London, London, W.C.* 1)

An individual has recently been described (Shooter, Skinner, Garlick & Barnicot, 1960) who possesses two haemoglobin variants Hb-G and

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Hb-G_2 in place of the normal haemoglobins Hb-A and Hb-A_2. A comparison of the electrophoretic mobilities of these haemoglobins led Gammack, Huehns, Shooter & Gerald (1960) to suggest that Hb-G_2 differs from Hb-A_2 in the same way as does Hb-G from Hb-A. Because Hb-A_2 is found in association with β-chain variants of Hb-A, then this shared abnormality of Hb-G and Hb-G_2 must reside in their α_2 subunits. This hypothesis also implies that Hb-A_2 has α_2^A subunits similar to Hb-A, the molecular formula of Hb-A_2 then being $\alpha_2^A\delta_2^{A_2}$, and of Hb-G_2, $\alpha_2^G\delta_2^{A_2}$. It has already been shown (Gammack *et al.* 1960) that Hb-G has abnormal α_2^G subunits and further recombination experiments have now given results consistent with these formulae (Huehns & Shooter, 1961).

Dissociation and recombination of isolated Hb-A_2 with Hb-G_α produced a new haemoglobin species migrating in starch gel more slowly than Hb-A_2 and in the position of Hb-G/C (Raper, Gammack Huehns & Shooter, 1960) as well as an increase in the Hb-A zone. Hb-G/C differs from Hb-C by the replacement of the α_2^A subunit of Hb-C with an abnormal α_2^G subunit and as Hb-C has the same mobility as Hb-A_2 it follows that the new haemoglobin could also differ from Hb-A_2 by the replacement of an α_2^A with an α_2^G subunit. The exchange reaction may therefore be written

$$\underset{\text{Hb-}G_\alpha}{\alpha_2^G\beta_2^A} + \underset{\text{Hb-}A_2}{\alpha_2^A\delta_2^{A_2}} \rightleftarrows \underset{\text{Hb-A}}{\alpha_2^A\beta_2^A} + \underset{\text{Hb-}G_\alpha}{\alpha_2^G\beta_2^A} + \underset{\text{Hb-}A_2}{\alpha_2^A\delta_2^{A_2}} + \underset{\text{Hb-G/}A_2}{\alpha_2^G\delta_2^{A_2}}$$

Difference in net charge from Hb-A

+2 +4 0 +2 +4 +6

No new species appeared, however, when Hb-A_2 or Hb-G_α by themselves or a mixture of Hb-A_2 and Hb-S were treated in the same way. When Hb-A_2 was dissociated and recombined with Hb-H, Hb-A was formed, indicating again that Hb-A_2 has α_2^A subunits similar to those of Hb-A. These results are therefore consistent with the formula $\alpha_2^A\delta_2^{A_2}$ for Hb-A_2. Examination of the tryptic peptides from Hb-A_2 support this conclusion (Stretton, 1960; Muller & Jonxis, 1960).

The study of individuals who carry four haemoglobins (Itano & Robinson, 1960; Raper *et al.* 1960) suggests that the last stage of haemoglobin synthesis is the combination of the independently produced α_2 and β_2 subunits. If this also applies to Hb-A_2, then the α_2^A subunits for the formation of Hb-A and Hb-A_2 could be derived from a common pool. It follows that an individual who synthesises abnormal α_2^x subunits will possess not only a variant of Hb-A, $\alpha_2^x\beta_2^A$, but also the corresponding variant of Hb-A_2, $\alpha_2^x\delta_2^{A_2}$. This appears to be so, for Hb-G_2, which replaces Hb-A_2, migrates at a similar rate to the doubly abnormal hybrid $\alpha_2^G\delta_2^{A_2}$ produced from Hb-A_2 and Hb-G_α *in vitro*. Finally if a person is synthesizing both α_2^A and α_2^G subunits then both Hb-A_2 and Hb-G_2 would be formed and this has been found in two unrelated carriers of the Hb-G_α trait.

We thank Dr H. Lehmann for a sample of Hb-G_α trait.

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(16) The Immunochemical Relationships Between the Components of Digested Human γ-Globulin with Rhesus-Monkey γ-Globulin

By D. S. Rowe (introduced by P. G. H. Gell). (*Department of Experimental Pathology, The Medical School, University of Birmingham*)

Human- and rhesus-monkey-serum γ-globulins cross-react (reaction of partial identity), as shown by double diffusion in agar, using specific antisera raised in rabbits against human γ-globulin. In order to locate the common determinant groups within the γ-globulin molecule, the antisera were absorbed with rhesus γ-globulin (serum), and then used for the analysis of digests of human γ-globulin, prepared by the method of Porter (1959).

Immunoelectrophoretic analysis showed that the absorption of an antiserum with a small amount of rhesus γ-globulin removed the antibody to the faster-migrating component of the digest, and that further absorption removed the antibody to the more slowly migrating component. In addition, this selective absorption was studied quantitatively in four different antisera, using the γ-globulins from three rhesus monkeys. After absorption, antibody was detected by double diffusion in agar against chromatographically separated fast and slow components of human γ-globulin. In every case it proved possible to absorb the antibody to the fast component of human γ-globulin by addition of less rhesus serum than that required to remove the antibody to the slow component. For a single antiserum the ratio of the amount of rhesus serum necessary for the absorption of slow-component antibodies to the amount required for absorption of fast-component antibodies was

similar with each of the three rhesus sera. For the four different antisera, the values of these ratios lay between two and four.

Such selective absorption was not achieved with human, chimpanzee and baboon sera.

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(17) Structural Reversion of Gelatin into a Collagen-type System Induced by Citrate

By A. COURTS. (*British Gelatine and Glue Research Association, London, N.* 7)

Previous work on gelatin-film infrared dichroism and gel optical rotation (Robinson, 1953), on electron microscopy of acetone-precipitated gelatin (Veis & Cohen, 1960) and viscometric and optical rotation studies of rat-tail-tendon soluble collagen (Flory & Weaver, 1960), have indicated that structural changes in gelatins may occur, under suitable conditions, leading to a partial reversion of randomly coiled molecules to the collagen fold.

The present work shows that the reversion of certain gelatins to a more ordered molecular structure is considerably enhanced by citrate, or similar buffer solutions. Thus, 0·25% gelatin in 0·15M-citrate (pH 3·7) at 20° gave an increase in η_{sp}/c from 0·7 to 5·6 dl./g. in 12 days compared with the change to 1·6 in aqueous HCl at the same pH. At pH 7 the changes of η_{sp}/c were 16-fold in citrate, two-fold in water. Although the viscosity of gelatin in citrate at zero time was constant in the range pH 2·2–10, the degree of increase in viscosity with time was strongly related to pH, giving the maximum effect at about pH 7.

The reversion phenomenon is unrelated to the weight-average molecular weight of the gelatin, but is directly related to the rigidity factor. Gelatins with rigidity values below 60 000 dynes/cm.^{2} [10° measurement, Saunders & Ward (1953)] gave only limited viscosity increases with time; above this value the degree of reversion was proportional to the rigidity. With high-rigidity materials (105 000 dynes/cm.^{2}) values of η_{sp}/c 12 dl./g. greater were obtained (20°, c = 0·25 g./dl.). Such values are similar to those reported for citrate-soluble collagens (Doty & Nishihara, 1958) and citrate-soluble eucollagen (Courts & Stainsby, 1959).

Further instances of collagen-type order in gelatin–citrate, not shown by gelatin–water systems under similar conditions, are (i) development of turbidity on dialysis or with M-NaCl, (ii) coprecipitation of fibres with chondroitin sulphate, (iii) fibre formation in 0·5M-KCNS [cf. soluble eucollagen; Courts (1961)], (iv) a well-defined denaturation temperature [cf. soluble collagens; Doty & Nishihara (1958)] and (v) a resistance at pH 7 to complete digestion by trypsin at 25°, with a partial retention of ordered molecular structure.

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(18) Studies on Urea and Heat Denaturation of Human Albumins

By P. CALLAGHAN and N. H. MARTIN. (*Department of Chemical Pathology, St George's Hospital Medical School, London, S.W.* 1)

Mackay & Martin (1957) demonstrated that on heating unstabilized serum albumin to 56° in 0·15M-NaCl, there was a split in the normal electrophoretic peak. Immunoelectrophoretic studies showed that both fractions still reacted with specific antisera (Houghton, unpublished data).

We have now used the technique of optical dispersion to obtain more information on this effect. The configurational changes when albumin was dissolved in increasing concentrations of urea have been examined and these compared with the effect of heat.

Jirgensons (1952) and Schellman (1958) showed that denaturation of protein is accompanied by a large increase in laevorotation while the dispersion constant (λ_c) approaches 220 mμ.

We found that urea concentrations below 2M gave no detectable change in optical dispersion of human albumin, but that there was a linear relation between further increases in urea concentration and the change in laevorotation and λ_c.

Estimations of the helical content based on Imahori's (1960) modification of the Moffitt equation suggested that native human albumin contained 40–50% right-handed α-helix which decreased to 5–10% in 9M-urea. Heating this solution caused complete denaturation

$$(\lambda_c = 220\ \mathrm{m}\mu,\quad a_0^s = 0,\ b_0^s = 0),$$

while heating albumin in 4M-urea gave constants similar to those for albumin in unheated 6M-urea ($\lambda_c = 243\ \mathrm{m}\mu$, $a_0^s = +140$, $b_0^s = -186$).

Parallel immunochemical studies using the Ouchterlony technique suggested that the progressive disorientation produced by urea modifies the reaction of albumin with specific antisera. The heating of albumin had a similar effect.

A 2% solution of human albumin in 0·15M-NaCl, after heating at 56°, had $\lambda_c = 255$ mμ compared with 265 mμ for unheated albumin. It was found possible to heat this albumin to 80° in distilled water without loss of solubility. The product consisted entirely of material with a lowered mobility, a minimal residual antigenic activity, and $\lambda_c = 240$ mμ, compared with that found in 9M-urea ($\lambda_c = 223$ mμ.).

Our studies suggest that the progressive rupture of hydrogen bonds by urea 'unwinds' the albumin molecule. The dispersion data from the heat-denatured product suggests that the situation is somewhat different, for while λ_c and b_0^* indicate that the molecule is about 50% unfolded, the values for specific rotation and a_0^* are not in line. Heating may produce refolding to give some β-form and α-helix, as suggested by Imahori (1960).

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(19) pH Dependence of the Fluorescence Yield of Tyrosine Copolymers

By K. Rosenheck and G. Weber. (*Weizman Institute of Science, Rehovoth, Israel, and Department of Biochemistry, University of Sheffield*)

Two copolymers, one (GT) containing 4% of tyrosine and 96% of glutamic acid, another (GLT) containing 4% of tyrosine, 58% of glutamic acid and 36% of lysine, were examined. The fluorescence spectra of both copolymers at all pH values were indistinguishable from that of tyrosine (emission maximum at 303 ± 1 mμ).

GT had a quantum yield $q = 0{\cdot}02$ at pH 9–6·5, both in salt-free solution and in M-NaCl. Below pH 5 the yield rose reaching $q = 0{\cdot}13$ without salt and 0·175 in M-NaCl, at pH 2. The behaviour of GT after *O*-methylation of the tyrosines with dimethyl sulphate was similar in the acid region but in the range of pH 4·5–11 the yield was over three times higher ($q = 0{\cdot}069$). From these observations and the quenching of the fluorescence of phenol and anisole by carboxylic acids (White, 1960) it is concluded that CO_2^- quenches the tyrosine fluorescence by accepting a proton from the tyrosine hydroxyl during the lifetime of the excited state yielding transitorily the non-fluorescent phenolate ion.

GLT showed low yield (0·003–0·03) at pH 10–6. In the isoelectric zone (pH 6–4) the yield rose to 0·14 at pH 5 and fell to 0·065 in the range 3·5–2. The high yield in the isoelectric zone was totally abolished by M-NaCl.

The findings in both polymers are explained on the assumption that the carboxyl groups are the main quenchers, exhibiting two modes of action; one as proton acceptors, and another independent of charge which is responsible for the yield of 0·069 in methylated GT at pH 11–4·5 and 0·062 in GLT at pH 3·5–2. Both modes of quenching require short-range interaction and may be decreased or abolished if the carboxyls are themselves involved in interaction with a group other than tyrosine.

The high yields of acid GT, where the extensive inter-carboxyl hydrogen bonding leads to aggregation of the polymer, may thus be explained, as well as the effect of salt which increases the extent of this bonding (Doty, Imahori & Klemperer, 1958). In the same way the high yield of isoelectric salt-free GLT is due to the binding of the CO_2^- by the charged lysines.

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(20) Tyrosine Fluorescence in the Albumins

By G. Weber. (*Department of Biochemistry, University of Sheffield*)

Examination of the fluorescence spectrum of proteins containing both tyrosine and tryptophan has shown no indication of tyrosine fluorescence (Teale, 1960). The use of the matrix method in the analysis of the spectrofluorometric data (Weber, 1961), shows the presence of two components and two components alone, in the fluorescence spectrum of human-serum albumin (HSA), bovine-serum albumin (BSA) and ovalbumin. From difference fluorescence spectra the second component may be shown to have a maximum of emission at 305 ± 3 mμ and a quantum yield of 0·018–0·022 in the three intact proteins. The second component is excited with a lesser efficiency than the main tryptophan fluorescence by wavelengths shorter or longer than 280 mμ and from this fact, and the emission spectrum, it may be identified as tyrosine. In solutions in 8M-urea the yield of the tyrosine fluorescence increases to 0·035 in HSA and 0·030 in BSA, while the fluorescence

yield of tryptophan declines to 0·058 and 0·096 from the original values of 0·080 and 0·163 respectively. Larger changes are seen in water solution after methylation of the phenolic hydroxyls of both proteins with dimethyl sulphate. The yield of tyrosine fluorescence is 0·058, that of tryptophan 0·036 in HSA, the corresponding values in BSA being 0·035 and 0·079. While some of the decrease in tryptophan yield may be explained by suppression of the electronic energy transfer from tyrosine, this can only have a very small effect in the increase in yield of tyrosine itself, most of which must come from the release of the interaction with a neighbouring quenching group. Experiments on the quenching of fluorescence of phenol and anisole by acids (White, 1960), the methylation experiments and the results reported by Rosenheck & Weber (1961) suggest this as the carboxylate ion. It must be stressed that although close proximity of the groups may be expected to occur during the lifetime of the exited state (1–3 μm-sec.) the existence of a hydrogen bond between the phenolic hydroxyl and the carboxylate ion is not explicitly required.

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(21) The Synthesis of Uniformly Labelled Ribonuclease by Slices of Ox Pancreas

By J. M. Barry. (*Department of Agriculture, University of Oxford*)

Vaughan & Anfinsen (1954) found that when slices of calf pancreas were incubated with labelled phenylalanine labelled ribonuclease was formed in which two different phenylalanine residues had different specific activities. Other proteins formed *in vitro* have also been unevenly labelled, while those formed *in vivo* have sometimes been evenly and sometimes unevenly labelled (see Kruh, Dreyfus & Schapira, 1960). It now seems clear that most proteins are formed by the reaction of amino acid derivatives on the surface of ribosomes, and although this mechanism is sometimes termed the 'simultaneous' linkage of amino acids, as opposed to reaction through free intermediate peptides, it must in fact occur by a series of distinct reactions. This being so, the most probable explanation of experiments in which evenly and unevenly labelled proteins have been formed is that the particular tissue, at the moment of contact with the labelled amino acids, has contained incomplete protein molecules of varying size which have been completed with labelled amino acids; the protein so formed would be unevenly labelled, provided the component amino acids are always incorporated into the molecule in the same order in time. This unevenness could be masked by evenly labelled protein formed subsequently.

If this theory is correct, degradation of an unevenly labelled protein could reveal the order in time in which its component amino acids are incorporated. Accordingly ox pancreas slices (12 g.) were incubated with L-[^{14}C]lysine (10 μc; 100 μg.) in a buffered medium containing glucose, for 2 hr. at 37° [roughly a repetition of the preparation of unevenly labelled ribonuclease by Vaughan & Anfinsen (1954) but with half their time of incubation and with glucose in the medium]. To ribonuclease A (6 mg.) isolated from the slices was added carrier ribonuclease (60 mg.); the mixture was oxidized, digested it with trypsin, and the resulting peptides separated by chromatography on Dowex-50 (Hirs, Moore & Stein, 1953, 1956). From these peptides were isolated eight samples of lysine from known positions in the protein chain. Their specific activities (averages of closely agreeing duplicates) varied from 4·01 to 4·27 with a mean of 4·14 μmc/mg. of lysine, and thus did not differ significantly. It is, therefore, clear that tissue slices do not always yield unevenly labelled proteins.

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(22) Polyribonucleotide Biosynthesis in the Nucleus and Cytoplasm of Mammalian Cells

By R. H. Burdon and R. M. S. Smellie. (*Department of Biochemistry, The University of Glasgow*)

Cytoplasmic extracts of ascites tumour cells, capable of polyribonucleotide biosynthesis, yield two soluble enzyme fractions catalysing the incorporation of uridine 5′-triphosphate (UTP), labelled with ^{32}P or ^{14}C, into polyribonucleotide (Burdon, 1960). With one of these preparations, cytoplasmic fraction A, the incorporation of UTP is greatly increased by the addition of a mixture of the 5′-triphosphates of adenosine, guanosine and

cytidine (ATP, GTP and CTP) and, under these conditions, most of the incorporated radioactivity is distributed in internucleotide linkages. The less extensive incorporation of labelled UTP catalysed by the other preparation, cytoplasmic fraction B, would appear to be terminal in nature and unaffected by the addition of a mixture of ATP, GTP and CTP, although it is greatly stimulated by a mixture of amino acids.

A soluble enzyme fraction, prepared in a manner similar to that for cytoplasmic fraction A, has been obtained from extracts of ascites cell nuclei. The incorporation of labelled UTP into polyribonucleotide catalysed by this preparation, nuclear fraction A, in the presence of added ascites cell ribonucleic acid (RNA), reduced diphosphopyridine nucleotide, $MgCl_2$, ATP, GTP and CTP, is substantially higher than the incorporation of UTP into polyribonucleotide by cytoplasmic fraction A, under identical conditions.

This chemical evidence would appear consistent with current views, based mainly on radioautography, that the main site of RNA synthesis is the cell nucleus, and also with the possibility that during the preparation of the cytoplasmic extracts of ascites cells, a small portion of the enzyme, or enzymes, responsible for RNA biosynthesis, is leached from the cell nuclei.

In addition, the incorporation of UTP into polyribonucleotide by both nuclear and cytoplasmic fractions A is greatly augmented by the addition, to the incubation medium, of RNA derived from ascites cell nuclei, whereas the addition of comparable amounts of RNA of cytoplasmic origin has a similar, but much less pronounced effect.

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(23) Countercurrent Ionophoresis

By J. C. Gage. (*Imperial Chemical Industries, Ltd., Industrial Hygiene Research Laboratories, Welwyn, Herts.*)

Countercurrent ionophoresis is a term applied to the concentration and separation of anions or cations from dilute aqueous solution. The solution is passed at constant rate through a cell across which is applied a d.c. potential sufficient to retain the required ions and to allow them to accumulate in the cell. The solution is followed by water at the same rate and with the same applied potential, when the ions arrange themselves in layers in order of their electrophoretic mobilities.

The potential is applied to the cell through ion-exchange membranes. To retain anions in the cell the solution enters at the anode end and the current is carried by ammonium ions from the anode compartment. To retain cations the cell is reversed and the current is carried by acetate ions from the cathode.

The cell is constructed from slotted Perspex plates bolted together and interleaved with filter paper, which enables the layers to be removed separately. From a knowledge of the rate of flow of the solution, the potential drop along the cell, the mobility of the current-bearing ion and the lengths of the layers, it is possible to calculate the number of equivalents of each ion present and their mobilities.

Examples of the way in which the cell has been used are the separation of acid dyes, and the isolation of radioactive metabolites from rat urine after administration of labelled trichloroethylene.

(24) On the Nature of Sterol Complexes in Bile and Intestinal Fluid

By J. C. Desai and J. Glover. (*Biochemistry Department, University of Liverpool*)

Previous studies have shown that dietary sterols mix rapidly and uniformly with endogenous sterols during absorption across the intestine (Glover & Green, 1957). It was presumed that they became molecularly dispersed in the lumen. A water-soluble sterol complex was detected in the supernatant fluid from the high-speed centrifugation of intestinal contents of fasting rats and animals dosed with 7-dehydrocholesterol (Glover & Stainer, 1958). These observations prompted us to make a comparison of this complex with that which occurs in bile (Verschure, 1956).

The intestinal complex is precipitated by half saturation with $(NH_4)_2SO_4$ or at pH 3. The crude precipitate contains all the sterols along with a little mucopolysaccharide and protein. The proportion of total lipids to sterols in the precipitate from fasting rats was 9–17:1 and that from rats dosed with 7-dehydrocholesterol 5–8:1. Portions of intestinal fluid concentrates from rats dosed with 7-dehydrocholesterol were subjected to either (*a*) electrophoresis or (*b*) centrifugation at 140 000*g* in media of different densities or (*c*) chromatography on diethylaminoethylcellulose powder. With procedure, (*a*) two zones, origin and the α-globulin region, were found to contain sterols. With (*b*), the complex was found to have a density between

1·03 and 1·22. It is strongly adsorbed or decomposed by diethylaminoethylcellulose and the sterols could be eluted with ethanol. None of the proteins which pass through the column carry sterols. A similar sterol complex was also obtained from ox-intestinal fluid.

Ox- and human-bile concentrates have also been fractionated electrophoretically. In agreement with the findings of Verschure (1956), most of the sterols from each moved in a compact zone with an ionic mobility 1·5 times that of albumin in borate buffer, pH 9·8. This zone is stained by the protein dye, azocarmine B, but not by naphthalene black 12B. The azocarmine-staining component however is extractable with ethanol. This lipid-containing zone also stains with toluidine blue and the periodic acid–Schiff reagent. The high ionic mobility together with the above suggests the presence of sulphated mucopolysaccharides. Bile salts are also present, the major component of which is the salt of deoxycholic acid. Fairly extensive dialysis and electrophoresis of the complex does not remove completely the deoxycholic acid salt. The proportion of bile salts to lipids in the original ox bile was about 10:1, whereas in the isolated complex it was about 1:2. The sterol content of the lipid fraction was constant at about 6–7%. The protein content of the complex based on the Folin-Ciocalteau test was approximately 1%. The polysaccharide moiety contained some hexose and hexuronic acid but only a small amount of hexosamine. The sterol complexes of bile and intestinal fluid appear to be different in many respects but both contain a polysaccharide moeity.

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(25) Configuration of the 6-Hydroxyl Groups in Phenolic Steroids Formed During Enzymic Reactions

By H. Breuer, R. Knuppen and Gerta Pangels. (*Chemische Abteilung, Chirurgische Universitätsklinik, Bonn-Venusberg, Germany*)

Recently, 6-hydroxylated phenolic steroids have been detected in a variety of species. Thus, liver of man (Breuer, Knuppen, Ortlepp, Pangels & Puck, 1960), rat (Breuer & Knuppen, 1960) and mouse (Mueller & Rumney, 1957) were found to metabolize oestradiol-17β to 6-hydroxyoestradiol-17β; the latter compound has also been isolated from the follicular fluid of the mare (Bush, Klyne & Short, 1960). When these experiments were performed the absolute configuration of the 6-hydroxyl groups in the reference compounds used was still unknown. Since it has now been possible to assign definitely the configuration to the two epimeric 6-hydroxyoestradiols (Breuer, Knuppen & Pangels, 1961) it seemed of interest to obtain more information about the configuration of the naturally occurring 6-hydroxylated oestrogens.

In all experiments, 200 μg. of steroid were incubated with 200 mg. of liver slices in 5 ml. of Krebs-phosphate saline for 60 min. at 37° in air. Tissue and saline were extracted with ether–chloroform (3:1, v/v) and the extracts subjected to paper chromatography. The products formed during incubation were identified by their paper-chromatographic behaviour and by various microchemical procedures.

The incubation of oestradiol-17β with rat-liver slices led to the formation of two phenolic metabolites (approximately 3% yield), more polar than oestradiol-17β, which could easily be separated from each other by chromatography in the system formamide/chloroform–ethyl acetate (5:1, v/v); the two compounds proved to be identical with 6α-hydroxyoestradiol-17β (mobility 14·7 cm./14 hr.) and 6β-hydroxyoestradiol-17β (mobility 12·8 cm./14 hr.). When 6α-hydroxyoestradiol-17β was incubated with human-liver or rat-liver slices 6-oxo-oestradiol-17β was formed; the incubation of 6-oxo-oestradiol-17β yielded only trace amounts of 6α-hydroxyoestradiol-17β. After the incubation of 6β-hydroxyoestradiol-17β with human-liver or rat-liver slices no 6-oxo-oestradiol-17β could be detected; under the same conditions 6-oxo-oestradiol-17β was reduced to 6β-hydroxyoestradiol-17β.

The results show that rat liver contains a 6α-hydroxylase and a 6β-hydroxylase which both attack phenolic steroids. In addition, a 6α-hydroxy steroid dehydrogenase and a 6β-hydroxy steroid dehydrogenase are present in rat liver as well as in human liver.

This investigation was supported in part by a grant from the Deutsche Forschungsgemeinschaft.

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(26) The Identification of Two 3β-Hydroxy 17-Oxo Steroids Isolated from the Urine of Female Guinea Pigs

By R. V. Brooks. (*Department of Chemical Pathology, St Thomas's Hospital Medical School, London, S.E.* 1)

Staib, Teller & Schmidt (1959) reported that the main 17-oxo steroid in the urine of female guinea pigs is a compound resembling 3β-hydroxy-5α-androstan-17-one (*epi*androsterone). Brooks, Clayton & Hammant (1960) independently confirmed this, but the material isolated by these latter authors had an infrared spectrum different from that of authentic epiandrosterone. It has now been shown that this material is an artifact formed during the isolation procedure.

It has been found that successive hydrolysis of the urine from female guinea pigs by β-glucuronidase followed by continuous extraction with ether at pH 0·8 releases very little 3β-hydroxy 17-oxo steroid. Subsequent hydrolysis with hot mineral acid (20 vol. % HCl, 100°, 20 min.) yields large quantities of the epiandrosterone-like compound. This material has been purified and found to have an infrared spectrum identical with that of $\Delta^{9:11}$-3β-hydroxyandrosten-17-one. When hydrolysis of the urinary steroid conjugates is carried out with dioxan–trichloroacetic acid or by the ethyl acetate solvolysis method of Burstein & Lieberman (1958), a more polar 3β-hydroxy 17-oxo steroid is obtained. This gives an ultraviolet absorption spectrum in conc. H_2SO_4 and a melting point identical with that of authentic 3β:11β-dihydroxy-5α-androstan-17-one. The infrared spectrum of the acetate is also identical with that of the authentic compound.

Relatively smaller quantities of the corresponding 11-oxo compound, i.e. 3β-hydroxy-5α-androstan-11:17-dione, have also been isolated from this source and identified by melting point, ultraviolet absorption spectrum in conc. H_2SO_4 and infrared spectrum. The significance of these findings in relation to the nature of the steroids secreted by the adrenal glands of the guinea pig was discussed.

The author is grateful to Dr A. E. Kellie for measuring the infrared spectra and to Professor W. Klyne for the authentic sample of 3β:11β-dihydroxy-5α-androstan-17-one from the Medical Research Council Steroid Reference Collection.

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(27) The Effects of Large Doses of Vitamin A Acid on the Bones of Young Rats

By J. N. Thompson and G. A. J. Pitt. (*Biochemistry Department, University of Liverpool*)

We have compared the activity of vitamin A alcohol (given as its acetate) with that of vitamin A acid (as the methyl ester) in producing hypervitaminosis A, as judged by the effect of excessive doses on the bones.

Twelve young male rats (wt. 64–103 g.) were divided into three groups of 4. In each group, one rat was given 2 mg. of vitamin A acid daily, another 4 mg. of vitamin A acid, and the third 8 mg. of vitamin A alcohol; the fourth served as a control. When the animal fed 4 mg. of vitamin A acid began to appear distressed, it was killed, together with all the animals in that group.

Many of the bones of the rats given vitamin A acid for 2 weeks were fractured, and all had undergone marked resorption (particularly on the higher dosage) when compared with those of the animals fed vitamin A alcohol. No fractures were found in those animals given vitamin A alcohol.

Vitamin A acid therefore induces much more severe signs of hypervitaminosis A than does vitamin A alcohol at twice or four times the dosage.

It is also possible with vitamin A acid to produce signs of hypervitaminosis A which have never been seen with vitamin A alcohol. For example, feeding weanling male rats 12 mg. of vitamin A acid over a period of 3 days caused distortion of the thoracic cage. Vitamin A alcohol in quantities up to 36 mg. produced no such effect.

This strongly hypervitaminotic effect of vitamin A acid is remarkable since vitamin A acid, unlike the alcohol, is not stored in the animal body and indeed disappears very rapidly. Also, the growth-promoting activity of vitamin A acid has never been found to exceed that of vitamin A alcohol.

We reconcile these findings in terms of a hypothesis put forward by Moore (1953). He suggested that vitamin A alcohol was oxidized to vitamin A acid, which could be the systemically active form (see also Dowling & Wald, 1960). Hypervitaminosis A induced by feeding vitamin A alcohol is probably not produced by the alcohol itself but by vitamin A acid formed from it, or a subsequent metabolite.

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(28) The Activity of Liver and Kidney Acid Phosphatase at Different Stages in the Life-Span of the Rat

By T. J. Franklin. (*Department of Biochemistry, University of Oxford*)

Information about the biochemical nature of the ageing process, particularly in relation to senescence, is limited. A study has been made of the activity of acid phosphatase, an enzyme associated with lysosomes and lysosome-like particles, in the liver and kidney at different stages in the life span of the rat. A measure of the distribution of the enzyme in the 'soluble' and 'bound' fractions has been obtained by taking advantage of the release of the enzyme from the particles in the presence of Triton X-100 (Wattiaux & De Duve, 1956). In 10- to 14-day-old rats the ratio of bound to soluble activity was found to be very high in both liver and kidney. Within a few weeks of birth the ratio had fallen to a level which remained essentially unchanged in animals aged 5–6 months and 12–14 months. In senile rats, aged 22–24 months, the ratio of bound to soluble activity was maintained at the young adult values in the liver but in the kidney there was a significant decrease in this ratio. Similar changes in the intracellular distribution of acid phosphatase and other lysosomal enzymes have been observed to be associated with the anoxia and resulting necrosis following ligation of the liver (De Duve & Beaufay, 1959) and with the degeneration of certain embryonic tissues (Brachet, DeCroly-Briers & Hoyez, 1958). It has been shown that ligation of the kidney also results in a decrease in the ratio of bound to soluble acid phosphatase activity. It is therefore suggested that the observed decrease in this ratio in the kidneys of senile rats may be associated with the well-known senescent degeneration of this organ (Kennedy, 1957).

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(29) Histological Variation as a Source of Biochemical Error

By J. Chayen, G. R. N. Jones, L. Bitensky and G. J. Cunningham. (*Department of Pathology, Royal College of Surgeons, London, W.C.* 2)

The enzymic activity of a homogenate of an organ will depend on the enzymic pattern of each kind of cell present and on the relative proportions of such cells. Thus changes produced by some external agent, such as a carcinogen, could be true alterations in metabolic pattern associated with change to a malignant condition; alternatively they might represent only a change in the proportions of the tissue elements. In many biochemical studies on the experimental induction of liver cancer in rats, it has been assumed that changes, such as reduced succinic dehydrogenase activity, were due to the former cause and the organ has been treated as a single homogeneous tissue. In fact, however, liver contains at least three tissues. It will be argued, therefore, that the more likely explanation of the data is that liver parenchyma cells maintain their activity, but that their effect is diluted by the great increase in proportion of otherwise normal cells of the other tissues in which this enzymic activity is very low.

The activity of succinic dehydrogenase in the liver of rats fed for various times with 4-dimethylaminoazobenzene has been studied. Samples of the liver were removed for histological and histochemical examination; the rest was homogenized and examined by the anaerobic methylene-blue technique. The reduction in activity during carcinogenesis has been confirmed, but qualitatively there was a good relationship between the fall in Q_{MB} and the degree of biliary hyperplasia. Both normal and hyperplastic bile duct cells showed very little succinic dehydrogenase activity histochemically (also see Pearson & Defendi, 1955), whereas that of the parenchyma cells remained high. In confirmation, even after prolonged feeding with the carcinogen, normal Q_{MB} values were found in regions which contained the normal proportions of cell types.

Hence in this study it is likely that the reduced succinic dehydrogenase activity was a tissue dilution artifact and that, as indicated histochemically, the carcinogen did not affect the activity of active parenchyma cells. Moreover it is probable that the low activity of the bile duct cells was not a premalignant change, but is a normal characteristic of this tissue and of its supporting fibrous tissue.

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Pearson, B. & Defendi, V. (1955). *Cancer Res.* **15**, 593.

(30) Historadiographic Identification of Alkaline Phosphatase

By A. J. Hale. (*Division of Pathology, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, W.C.* 2)

There have been few reports of attempts to measure enzyme activity cytochemically because

of lack of information about the physical characteristics of reaction products and inability to measure the products accurately (Hale, 1961). Barter, Danielli & Davies (1955) used the interference microscope to measure the activity of alkaline phosphatase, but they pointed out that they could not measure the rate of liberation of phosphate ions, merely the rate of deposition of calcium phosphate of unknown form, at the site of activity.

Much is known about the specific absorption coefficients at ultra-soft X-ray wavelengths of calcium salts and thus they can be identified and measured in cells using radiohistographic methods (Engström, 1958). Using a simple radiohistograph (Engström & Lundberg, 1957) the author has studied the deposition of calcium phosphate at the site of activity of alkaline phosphatase. Frozen sections of rat kidney were incubated for varying times at 37° in a substrate containing sodium β-glycerophosphate and calcium chloride. After incubation, sections were mounted on Kodak maximum resolution plate and dried. The specimens were then exposed for 60–90 min. at 1·5 kv and 1·5 mA. Heavy absorption of X-rays occurs at the free border of the cells lining the convoluted tubules. Selection of suitable wavelengths permits measurements of the amount of phosphorus liberated by the enzyme under different conditions.

It is possible to examine the localization of elements of high atomic number (> 14) which are deposited in tissues by 'staining' reactions (Hale, 1958). Consequently a study has been made of the changes of localization produced by converting calcium phosphate into cobalt phosphate and eventually into cobalt sulphide, as in the classic Gomori-Takamatsu reaction.

Selection of suitable X-ray wavelengths will permit measurement of the absolute amounts of other elements, thus this technique can be used to study the kinetics of other enzymically controlled reactions at the microscopic level.

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(31) Peptidase Activity of Normal and Diseased Human Gastrointestinal Mucosal Cells

By W. H. TAYLOR. (*Department of Chemical Pathology, The United Liverpool Hospitals, Liverpool* 3)

Normal human gastric fundic mucosal extracts vary in their ability to hydrolyse dipeptides and tripeptides. Some subjects were without such activity; in others, DL-alanylglycine was split maximally at pH 6·8 and glycylglycylglycine at pH 7·2, although hydrolysis of the latter substrate was always slight. Pyloric mucosal extracts show a similar variation and although some attack DL-alanylglycine readily, glycylglycylglycine is only hydrolysed with difficulty. The responsible enzymes are not secreted into normal human gastric juice (Taylor, 1956).

Mucosal extracts from all parts of the human small and large intestine possess peptidase activity. Both DL-alanylglycine and glycylglycylglycine are hydrolysed fairly readily. The pH–activity curves for digestion of glycylglycylglycine are similar and show a maximum at pH 8·2, but those for DL-alanylglycine, from different parts of the intestine, vary.

In patients with carcinoma of the stomach, extracts of the uninvaded fundic mucosa digest DL-alanylglycine and glycylglycylglycine more readily than do extracts of mucosa from normal subjects and, with the former substrate, usually exhibit two pH maxima, at pH 7·0–7·6 and at pH 8·2–8·5. Extracts of the carcinomata hydrolyse DL-alanylglycine readily but have little or no action on glycylglycylglycine. The pH–activity curves for hydrolysis of DL-alanylglycine are sometimes identical with those obtained with uninvaded gastric mucosa from the same patient but sometimes differ markedly.

Extracts of carcinomata of the colon and rectum hydrolyse both DL-alanylglycine and glycylglycylglycine readily. The pH–activity curves for the latter substrate resemble those obtained with uninvaded mucosal extracts, in showing maximal digestion at pH 8·2. A similar resemblance is not always seen with DL-alanylglycine.

Gastric juice from patients with peptic ulcer has no peptidase activity (Taylor, 1956). Fundic mucosal extracts from such patients usually hydrolyse DL-alanylglycine and glycylglycylglycine more readily than do comparable extracts from normal subjects, but are occasionally inactive. The pH–activity curves of the active extracts, and the pH maxima, vary widely. Pyloric extracts from patients with peptic ulcer also possess higher peptidase activity than do those from normal subjects. The difference is particularly marked in the hydrolysis of glycylglycylglycine. The pH maxima for the digestion of both di- and tripeptides also differ from those obtained with normal pyloric extracts.

The significance of these differences was discussed.

This work was in part assisted by a grant from the Medical Research Council.

Taylor, W. H. (1956). D.M. Thesis. University of Oxford.

(32) Purification and Properties of Liver Glutamic–Alanine Transaminase from Normal and Corticoid-injected Rats

By H. L. SEGAL and DIANA S. BEATTIE. (*Department of Pharmacology, St Louis University School of Medicine, St Louis, Mo., U.S.A.*, and *Department of Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pa, U.S.A.*)

Glutamic–alanine transaminase has been purified from normal rat liver to a specific activity of 20 μmoles of pyruvate formed/min./mg. of protein, by means of selective heat and acid denaturation, ammonium sulphate fractionation and gel adsorption and elution, representing a 100- to 200-fold enrichment from whole liver. The same degree of purification was achieved from livers of corticoid-treated rats, in which the initial specific activity is about five times that of the normal (Rosen, Roberts & Nichol, 1959). After incubation of the purified enzyme with α-oxoglutarate and a variety of amino acids, formation of glutamic acid could be detected only with alanine, and to a much lesser extent, valine.

In addition to the similarity of behaviour of the enzymes from normal and corticoid-treated rats in the purification procedure, the purified preparations exhibited the same pH optimum in 2-amino-2-hydroxymethylpropane-1:3-diol buffer and the same apparent Michaelis constants for alanine and α-oxoglutarate. Values of the apparent Michaelis constant for alanine were 3·7, 17·5 and 22·0 mM at α-oxoglutarate concentrations of 2·5, 6·7 and 16 mM respectively, and for α-oxoglutarate were 0·36 and 0·84 mM at alanine concentrations of 16 and 48 mM respectively, with the normal enzyme.

Simultaneous administration of ethionine with cortisone prevented to a large extent the increase in enzyme activity obtained with corticoids alone.

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(33) Repression of Cystathionase Production in *Escherichia coli*

By R. J. ROWBURY and D. D. WOODS. (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The properties of induced mutants of various micro-organisms suggest that cystathionine is an intermediate in the biosynthesis of methionine and is the immediate precursor of homocysteine (Davis, 1955). Wijesundera & Woods (1953) obtained a cell-free preparation of a cystathionase from *Escherichia coli* which degraded cystathionine to homocysteine, pyruvate and ammonia. It is now found that ultrasonic extracts of *E. coli* PA15 (a serine or glycine auxotroph) also contain cystathionase; when dialysed and treated with Dowex-1 resin pyridoxal phosphate is required for maximum activity.

The cystathionase activity of ultrasonic extracts decreased progressively as increasing concentrations of DL-methionine were added to the growth medium. The activity of homocysteine methylase is also reduced when methionine is present during growth (Rowbury & Woods, 1961) and in the present work the relative decreases in the two enzymes were similar; methionine (10 mM) repressed cystathionase by 80% and homocysteine methylase by 85%. These observations are a further example of repression of enzyme formation by the ultimate product of a sequence of reactions and may also be another case of 'co-ordinate repression' (Ames & Garry, 1959). Growth in the presence of DL-homocysteine (5 mM) did not significantly affect cystathionase production.

The cystathionase activity of ultrasonic extracts of organisms grown with methionine increased rapidly when the intact organisms were resuspended in a methionine-free medium; it reached within 3 hr. the level obtained with organisms originally grown without methionine. Total enzymic activity increased throughout relatively more rapidly than total mass of organisms and in the first hour trebled without appreciable growth; the change is probably therefore a phenotypic one. Evidence that recovery from repression requires *de novo* synthesis of protein comes from the observed inhibition of the process by chloramphenicol. Cystathionase and homocysteine methylase activities increased at a similar rate in the methionine-free medium.

Ultrasonic extracts of several strains of *E. coli* also metabolized D-serine and L-cysteine with formation of pyruvate. Previous growth in the presence of methionine did not affect production of pyruvate from D-serine but reduced that from L-cysteine by about 85% (10 mM-methionine). This reaction does not occur in the biosynthetic pathway to methionine; there is evidence however that cystathionase itself also deaminates L-cysteine.

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(34) Studies on a Macromolecule Isolated From *Shigella flexneri* and Acting as Phage Receptor

By J. Dirkx and J. Beumer. (*Institut Pasteur, Bruxelles, Belgium*)

Phages are known to adsorb on to specific sites of the bacterial surface. Such sites, which are called receptors, have been isolated in a number of cases (Goebel & Jesaitis, 1952; Weidel, Koch & Bobosch, 1954; Beumer & Dirkx, 1960*a*). The present work deals with the isolation and characterization of a new receptor involved in the H-F6S phage–*Shigella flexneri* F6S system. The receptor substance is isolated by extracting the bacteria with 5% trichloroacetic acid at 4° for 3 hr. The supernatant is precipitated with 3 vol. of 94% ethanol at −10 overnight. The precipitate, dissolved in water, is dialysed for 5 days against distilled water and reprecipitated under the same conditions. Purification of the receptor is performed through digestion by crystallized proteolytic enzymes: first pepsin at pH 2·0 for 1 day followed by trypsin at pH 8·6 for 2 days. Deproteinization is completed by shaking with amyl alcohol and chloroform. The substance is finally reprecipitated and dried under vacuum. It is homogeneous by electrophoresis and diffusion. The material contains 1·37% N and 1·38% P and was found to consist of two-thirds polysaccharide and one-third lipid. The polysaccharidic moiety contains glucose (38·5%), rhamnose (21·6%) and glucosamine (18·6%); neither 3:6-dideoxyhexoses nor heptoses have been found.

The molecular weight, as determined by light scattering, is approximately 47×10^6. Adopting for the molecule the random coil model, the mean diameter of 148 mμ has been calculated and found consistent with ultrafiltration data. Treatment of the light-scattering measurements following Timasheff & Townend (1960) has demonstrated dimerization of the molecule in water solution, the association constant being 12×10^6 l./g.

Diffusion measurements performed at 1° in distilled water give the extremely low value of $1{\cdot}91 \times 10^{-8}$ cm.²/sec.

Electrophoresis experiments show one single peak, the mobility of which remains practically nil in the pH range 2·5–10·0.

The receptor substance may be chemically titrated in the bacteria by using specific reactions for rhamnose (Dische & Shettles, 1948) or glucosamine (Dische & Borenfreund, 1950). Both methods give reasonably concordant results and lead to assume the presence of about 670 molecules of receptor substance per bacterium.

The biological activity of this substance is high; as little as 0·5 μg./ml. inactivates 50% of a phage suspension containing 2×10^6 particles/ml. In addition to phage H-F6S the material also exhibits receptor activity for phages H-SHPB, H+ and V. Cationic requirements for absorption of those phages on the isolated receptor have been found to be very specific in some cases and closely similar to the ones demonstrated previously for adsorption on to living bacteria (Beumer & Dirkx, 1960*b*).

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(35) The Rôle of Phospholipids in Protein Biosynthesis

By G. N. Godson and G. D. Hunter. (*Chester Beatty Research Institute, London, S.W.* 3)

Recent work in our laboratory (Hunter, Brookes, Crathorn & Butler, 1959; Hunter & Goodsall, 1961) has shown that labelled amino acids are rapidly bound in lipid fractions when they are incubated under suitable conditions with protoplasts of *Bacillus megaterium*. The evidence obtained also suggested that the lipo–amino acid complexes obtained might play some part in the process of protein biosynthesis.

Further studies (Godson, Hunter & Butler, 1961) have now been carried out on the labelling of protein fractions when protoplasts are incubated with ^{14}C-labelled amino acids for very short time periods down to a few seconds. At the earliest times studied, labelled protein is found to be distributed between a ribonucleoprotein or ribosomal fraction and a phospholipoprotein fraction (containing also about 2% of ribonucleic acid). The greater part of the labelled protein is bound in the phospholipoprotein fraction, and does not seem to be derived from that occurring in the ribosomes. It is possible that the template for protein biosynthesis contains phospholipid as well as ribonucleic acid (see Hunter & Godson, 1961); this possibility was discussed.

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DEMONSTRATIONS

Countercurrent Ionophoresis

By J. C. Gage. (*Imperial Chemical Industries Ltd., Industrial Hygiene Research Laboratories, Welwyn, Herts.*)

The separation of three acid dyes by countercurrent ionophoresis was demonstrated. A continuous desalting apparatus was shown for the removal of sodium and chloride ions from weaker acids prior to separation in the cell.

An Apparatus for Maintaining a Range of Constant Temperatures

By M. J. Selwyn. (*Department of Biochemistry, University of Cambridge*)

The apparatus has been designed to facilitate the study of enzyme stability and kinetics over any desired range of temperatures. Two constant-temperature baths (one of which can often be an ice bath) are used to maintain a temperature gradient along a well lagged brass bar; test tubes placed in holes drilled in the bar rapidly attain the temperature at this point of the bar, provided that water is placed in the holes to ensure good thermal contact between the bar and the test tubes.

To reduce the flow of heat along the bar and to produce a series of discrete temperature steps rather than a continuous gradient, the bar is made of brass blocks, each with a hole for a test tube, interspaced with 1·5 mm. thick plates of Tufnol, a material of low thermal conductivity. The Tufnol and brass blocks are cemented together with Araldite resin adhesive and the brass blocks at each end of the bar are soldered into the water baths. Thermal insulation is provided by cementing a 5 cm. deep Bakelite block on top of the bar with holes matching those in the brass bar and mounting the bar with attached water baths in a box containing insulating material.

The apparatus demonstrated provides eleven different temperatures and when used over the range 0–50° gives an evenly spaced series with each temperature constant to within 0·5° when the room temperature does not vary by more than 8°. Greater constancy of temperature could be achieved by reducing the insulation between the holes but this would increase the gradient across the holes and necessitate replenishing the ice bath more frequently than in the case of the apparatus shown, which can be left working overnight without attention.

The author thanks Dr M. Dixon, F.R.S., and Dr E. C. Webb for their advice and encouragement, and the department workshop staff for assistance in the construction of the apparatus. This work was done during the tenure of a D.S.I.R. Research Studentship.

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ADP, CDP, GDP, IDP, UDP	5′-Pyrophosphates of adenosine, cytidine, guanosine, inosine and uridine
AMP etc.	Adenosine 5′-phosphate etc.
ATP etc.	Adenosine 5′-triphosphate etc.
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
DPN	Diphosphopyridine nucleotide
EDTA	Ethylenediaminetetra-acetate
FMN	Flavin mononucleotide
FAD	Flavin–adenine dinucleotide
GSH, GSSG	Glutathione, reduced and oxidized
NAD	Nicotinamide–adenine dinucleotide
NADP	Nicotinamide–adenine dinucleotide phosphate
NMN	Nicotinamide mononucleotide
RNA	Ribonucleic acid
TPN	Triphosphopyridine nucleotide
tris	2-Amino-2-hydroxymethylpropane-1:3-diol

Abbreviations for amino acids and carbohydrates. Those marked * and † in *Suggestions to Authors* are to be used only for representation of polymers or sequences and in Tables and Figures.

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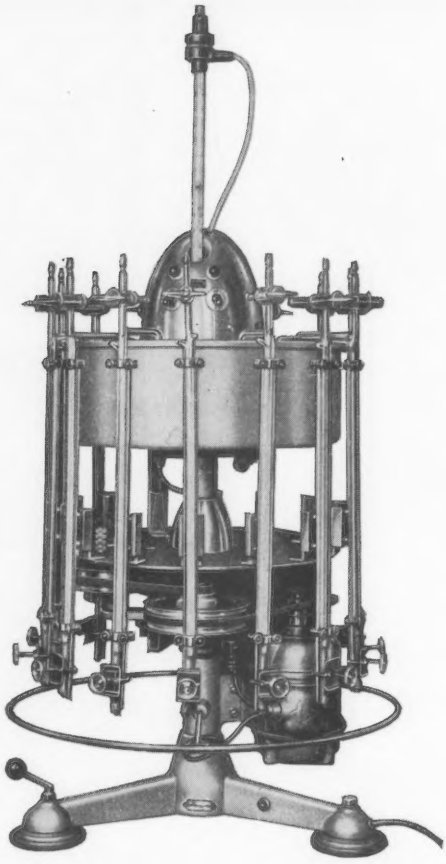

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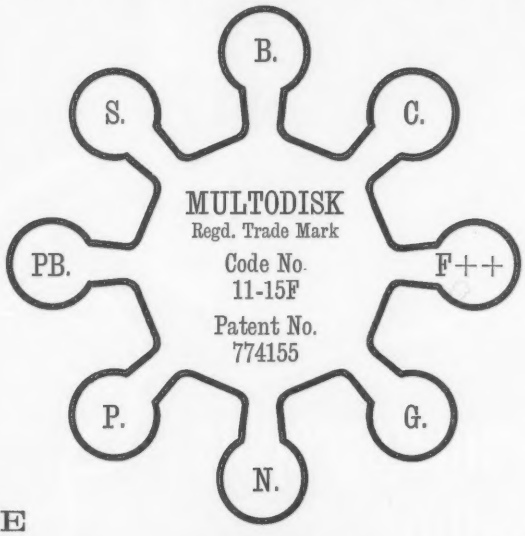

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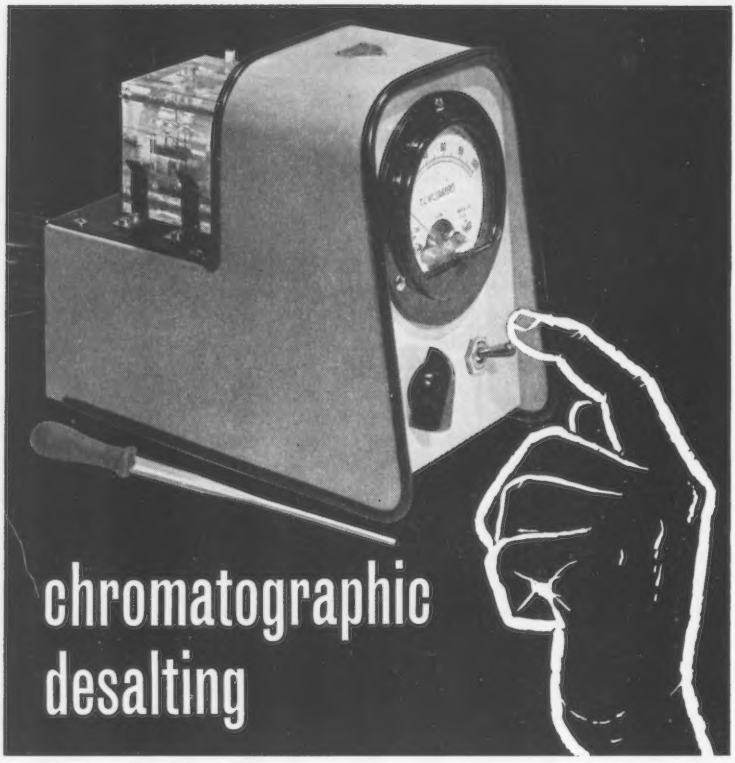

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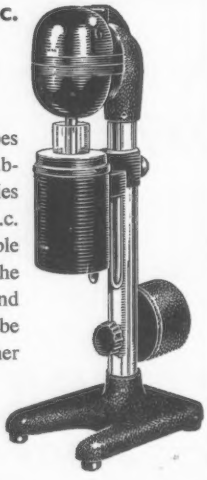

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